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DISORDERS OF MITOCHONDRIAL DNA POLYMERASE: A CLINICAL, BIOCHEMICAL AND STRUCTURAL STUDY

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ACADEMIC DISSERTATION

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This thesis is based on the following publications:

I Palin EJH, Hakonen AH, Korpela M, Paetau A, Suomalainen A. Mitochondrial recessive ataxia syndrome mimicking dominant spinocerebellar ataxia.
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II Palin EJH, Paetau A, Suomalainen A.
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Submitted

III Palin EJ, Lesonen A, Farr CL, Euro L, Suomalainen A, Kaguni LS
Functional analysis of H. sapiens DNA polymerase gamma spacer mutation W748S with and without common variant E1143G.
Biochim Biophys Acta 2010; 1802: 545-51.

IV Euro L*, Farnum GA*, Palin E, Suomalainen A, Kaguni LS. Clustering of Alpers disease mutations and catalytic defects in biochemical variants reveal new features of molecular mechanism of the human mitochondrial replicase, Pol γ.
Nucleic Acids Research 2011; 39: 9072-9084.

The publications are referred to in the text by their roman numerals.

In addition, some unpublished data is presented.

* Equal contribution

1 ABBREVIATIONS

Acetyl-CoA	Acetyl coenzyme A
ADP	Adenosine diphosphate
adPEO	Autosomal dominant PEO
AID	Accessory interacting determinant (subdomain in POLG1)
AMP	Adenosine monophosphate
ANT1	Adenine nucleotide translocator
APU	Average processed unit
ARJPD	Autosomal recessive juvenile Parkinson's disease
arPEO	Autosomal recessive PEO
ARSACS	Autosomal recessive spastic ataxia of Charlevoix-Saguenay
ATP	Adenosine triphosphate
BP	Base pair
CI	Respiratory chain complex I
CII	Respiratory chain complex II
CIII	Respiratory chain complex III
CIV	Respiratory chain complex IV
COX	Cytochrome c oxidase
DNA	Deoxyribonucleic acid
DNM1L	Dynamin-1-like protein
dNTP	Deoxyribonucleotide
dRP	5'-deoxyribose phosphate
dsDNA	Double stranded DNA
ER	Endoplasmic reticulum
ETC	Electron transport chain
IOSCA	Infantile-onset spinocerebellar ataxia
IP	Intrinsic processivity (subdomain in POLG1)
LRRK2	Leucine-rich repeat kinase 2
LSP	Light strand promoter
MAM	Mitochondria associated endoplasmic reticulum membrane
MELAS	Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes
MFN1	Mitofusin 1
MFN2	Mitofusin 2
Mip1	Yeast mitochondrial DNA polymerase
MIRAS	Mitochondrial recessive ataxia syndrome
MNGIE	Mitochondrial neurogastrointestinal encephalopathy
MRI	Magnetic resonance imaging
mtDNA	Mitochondrial DNA
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtSSB	Mitochondrial single-stranded DNA binding protein
NAD+	Nicotinamide adenine dinucleotide

nDNA	nuclear DNA
nt	Nucleotide
OPA1	Optic atrophy 1
OXPHOS	Oxidative phosphorylation
PD	Parkinson's disease
PEO	Progressive external ophthalmoplegia
Pi	Inorganic phosphate
PINK1	PTEN-induced putative kinase protein 1
POLG	Mitochondrial DNA polymerase / DNA polymerase gamma
POLG1	Mitochondrial DNA polymerase, catalytic subunit
POLG1-PD	PEO with parkinsonism, caused by POLG1 mutations
POLG2	Mitochondrial DNA polymerase, accessory subunit
POLRMT	Mitochondrial RNA polymerase
ptDNA	primer-template DNA
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RRF	Ragged red fibers
RRM2B	Ribonuclease reductase 2 B
SANDO	Sensory ataxic neuropathy, dysarthria and ophthalmoparesis
Sirt1	Sirtuin1
SN	Substantia nigra
ssDNA	Single stranded DNA
T7 GP4	T7 gene product 4 primase-helicase
TCA	Tricarboxylic acid cycle
TFAM	Transcription factor A, mitochondrial
TFB2M	Transcription factor B2, mitochondrial
TK2	Thymidine kinase 2

2 ABSTRACT

Mitochondrial dysfunction is a common cause of hereditary neurodegenerative diseases, such as Parkinson's disease and inherited ataxia syndromes. Frequent causes of mitochondrial dysfunction are mutations in mitochondrial DNA (mtDNA) or mutations in the genes encoding the mtDNA replication machinery. The minimal mtDNA replication machine consists of mitochondrial DNA polymerase, named DNA polymerase gamma (POLG), TWINKLE helicase and mitochondrial single-stranded DNA binding protein (mtSSB). Mutations in the gene *POLG1*, which encodes the catalytic subunit of POLG, cause a heterogeneous group of disorders with varying clinical phenotypes, such as mitochondrial recessive ataxia syndrome (MIRAS), progressive external ophthalmoplegia (PEO) and Alpers' syndrome. MIRAS is the most common inherited ataxia in Finland and a frequent form of inherited ataxia in Norway and Belgium. Sequence variants in *POLG1* also alter the risk of developing idiopathic Parkinson's disease. The cause of variability in the clinical manifestations is unknown.

Our results indicate that respiratory chain complex I (CI) deficiency does not explain parkinsonism in POLG1-PD patients and that mesencephalic alterations are a common feature in patients with dysfunctional mtDNA replication machinery. We have also elucidated the pathogenic mechanisms of MIRAS, from the molecular alteration of POLG1 function to the neuropathological changes and clinical phenotype, and thus our results help to understand the varying clinical manifestations associated with *POLG1* mutations. In addition, we have produced a classification of *POLG1* mutations, which could be useful in the evaluation of the potential pathogenicity of new *POLG1* mutations.

In this thesis, we studied the clinical picture of MIRAS. The high carrier frequency of the MIRAS disease allele in Finland, Norway and Belgium may lead to multiple introductions of the disease allele and some MIRAS cases to be mistakenly recognised as dominantly inherited, thereby leading to the exclusion of MIRAS as the diagnosis. We described a family in which both mother and son had MIRAS-like disease. We found that the father was a carrier of the MIRAS disease allele. Thus, MIRAS should not be ruled out by an apparently dominant mode of inheritance in countries with high carrier frequency of the disease allele.

Patients with PEO caused by POLG1 mutations have variable clinical manifestations, including premature menopause and parkinsonism (POLG1-PD). The cause of this variability is unknown. Also, the variability in clinical features is more evident in POLG1 diseases: diseases caused by malfunction

of other mitochondrial DNA replication proteins cause usually clear clinical phenotypes, with no such variation. We studied the pathogenic changes leading to parkinsonism by performing a neuropathological examination of the mesencephalon of two PEO patients with parkinsonism and by comparing the changes to patients with MIRAS, infantile-onset spinocerebellar ataxia (IOSCA) or PEO caused by mutations in *C10ORF2*, the gene encoding TWINKLE. POLG1-PD patients had an almost complete loss of neurons in substantia nigra (SN) whereas MIRAS, IOSCA and Twinkle PEO patients had also mild to moderate neuronal loss in the SN. POLG1-PD patients had also moderate loss of neurons in the oculomotor nucleus. CI activity was clearly reduced in the SN of all patients. The parkinsonism in POLG1-PD patients is in our view secondary to the drastic neuron loss in the mesencephalon and the lack of CI activity is a general feature of diseases caused by dysfunctional mitochondrial DNA replication and it does not correlate with parkinsonism, age or phenotype of the patients.

We studied the biochemical background underlying MIRAS and found that p. Trp748Ser POLG1 does not exhibit a biochemical phenotype in DNA polymerase function. Our findings challenge previously published results and their implications. We biochemically characterized recombinant POLG1, with the MIRAS causing amino acid change: p.Trp748Ser. The mutant POLG1 did not have any decrease in DNA polymerase activity on various primer-template DNAs or in the subunit interaction with the accessory subunit. There was a minor decrease in the DNA binding capability of the p.Trp748Ser POLG1, but this did not alter the overall efficiency of the enzyme as measured in the processivity.

We examined the distribution of Alpers' syndrome causing mutations on the protein structure of POLG1. We found that the mutations can be divided into five distinct groups, each with a different biochemical profile. Our findings were supported by the published biochemical characterizations of various mutant POLG1 enzymes. Based on the structural data, we predict that the p.Trp748Ser amino acid change affects the interaction with mtSSB and not intrinsic DNA polymerase activity or DNA binding of POLG1, which could explain the results from biochemical characterization.

3 INTRODUCTION

One essential challenge of life is reproduction. This requires that we grow and survive the daily obstacles. A major obstacle revolves around food, or more specifically, energy, which we need in order to survive. Humans have evolved to efficiently use various sources of stored energy: plants, which have stored energy produced by the sun and meat, into which energy stored by plants is processed. We can divide the various energy stores into carbohydrates, proteins and fatty acids. The elegant process that breaks down these energy stores efficiently into adenosine triphosphate, the common unit of energy used by our cells, revolves around mitochondria. Mitochondria are cellular organelles that are essential for our energy metabolism. They have their own genome, which has given evolution the possibility to experiment with more complex systems that use energy more efficiently and ultimately produce higher organisms, such as humans.

The proper function of mitochondria is important for the well-being of a cell and in the end, the whole organism. Dysfunctional mitochondrial can lead to disease. Mitochondrial diseases are a heterogeneous group of diseases, ranging from metabolic diseases such as diabetes mellitus to neurodegenerative disorders such as Parkinson's disease. Neurodegenerative phenotypes produce the most dramatic clinical manifestations and devastating consequences for patients. Our brain continuously requires enormous amounts of energy and even a small disruption in this can cause neuronal death, which after a certain threshold may lead to disease. Every neuron type has its own characteristics and energy requirements, whereas every disorder challenges different parts of the nervous system. These features at least in part contribute to the great heterogeneity of mitochondrial disorders.

Mitochondrial diseases are diseases of energy metabolism. Mitochondrial DNA encodes the most conserved core components of the oxidative phosphorylation enzyme complexes required for efficient energy metabolism and disorders of mitochondrial DNA or its replication disrupt this elegant process. In this thesis, we have studied the malfunction of mitochondrial DNA replication, caused by mutations in the mitochondrial DNA polymerase. Even among the various clinical manifestations caused by mitochondrial disease, malfunction of mitochondrial DNA polymerase leads to an even more diverse set of clinical manifestations.

4 REVIEW OF THE LITERATURE

4.1 THE EVOLUTIONARY ORIGINS OF MITOCHONDRIA

The Endosymbiotic Theory is the most widely accepted theory for the origin of mitochondria and it hypothesises that mitochondria evolved from a single event in which an alpha-protobacterium was introduced into a proto-eukaryotic cell, possibly the same event as when eukaryotes themselves evolved (Lane and Martin, 2010; Martin and Muller, 1998). Working as the power plant of the cell, it most likely allowed eukaryotes to evolve further and evolution to experiment with a far larger genome as was previously possible (Lane and Martin, 2010). Since the inception of eukaryotes and mitochondria their symbiosis has remained the same, but the appearance of participants has taken many forms: a wide array of different eukaryotes exists, from simple baker's yeast *Saccharomyces cerevisiae* to primates such as humans. Also, mitochondria are not equal in appearance as their size, organization and genome vary (Anderson et al., 1981; Palmer et al., 1992).

4.2 MITOCHONDRIA AS A MEMBER OF THE CELL

Cell organelles do not function alone or without context. Most of the proteins mitochondria require to function are encoded by nuclear genes. Furthermore, there seems not to be a population of disconnected mitochondria in most cells but rather a mitochondrial network which encompasses the whole cell (Lewis and Lewis, 1914; Palade, 1953). This network is dynamic and is constantly changing its shape and composition. Again, this requires help from other intracellular organelles, namely the endoplasmic reticulum or more specifically, mitochondrial associated endoplasmic reticulum membrane (MAM).

MAMs function as contact sites between mitochondria and endoplasmic reticulum and they seem to be more common than previously thought (Mannella et al., 1998; Rizzuto et al., 1998). They help in direct transfer of lipids between mitochondria and endoplasmic reticulum, Ca^{2+} influx, apoptosis and mark the sites of mitochondrial division (de Brito and Scorrano, 2008; Friedman et al., 2011; Gaigg et al., 1995; Garcia-Perez et al., 2008; Simmen et al., 2005). The MAM is held together with the help of protein tethering complexes, of which mitofusin 2 (MFN2) is the major component (Garcia-Perez et al., 2011).

4.3 MITOCHONDRIAL NETWORK

No strict definition of a mitochondrial network exists, but every mitochondrion can be considered to be a part of the same network within one cell. The constant flux in fission and fusion of mitochondria is mediated by mitofusin I, II and optic atrophy I proteins (MFN1, MFN2 and OPA1, respectively) in fusion and dynamin 1-like protein (DNM1L) in fission (Figure 1) (Cipolat et al., 2004; Smirnova et al., 2001). MFN1 and MFN2 are homologues of yeast Fzo1p, which coordinate the fusion of the mitochondrial membranes whereas OPA1 regulates this interaction via MFN1. The dysfunction of fusion or fission results in highly abnormal mitochondria, especially in post-mitotic tissues such as neurons: loss of fission in neuronal specific *DNM1L* knock-out mice decreases the amount of neurites and leads to dysfunctional synapse formation, whereas a decrease in fusion results in mtDNA loss, mitochondrial motility defects and an increase in reactive oxygen species (ROS) production (Chen et al., 2003; Ishihara et al., 2009; Parone et al., 2008). Instead of placing the burden of environmental stressors on individual mitochondria, fusion and fission seem to divide the burden to the whole network (Chen et al., 2010). The clinical phenotypes caused by the dysfunction of fusion by mutations in *MFN2* lead to Charcot-Marie Tooth disease 2A, a disease characterized by onset in early adulthood, neuropathy and peripheral muscle atrophy (Zuchner et al., 2004). Mutations in *OPA1* lead to dominant optic atrophy, which causes optic neuropathy and ultimately distortion of vision (Alexander et al., 2000; Delettre et al., 2000). As a further confirmation of their relevance to the same pathway, the symptoms resemble each other and overlap in these diseases. Mutations in *DNM1L* are also of clinical importance as they cause early infant mortality and cardiomyopathy (Ashrafian et al., 2010; Waterham et al., 2007).

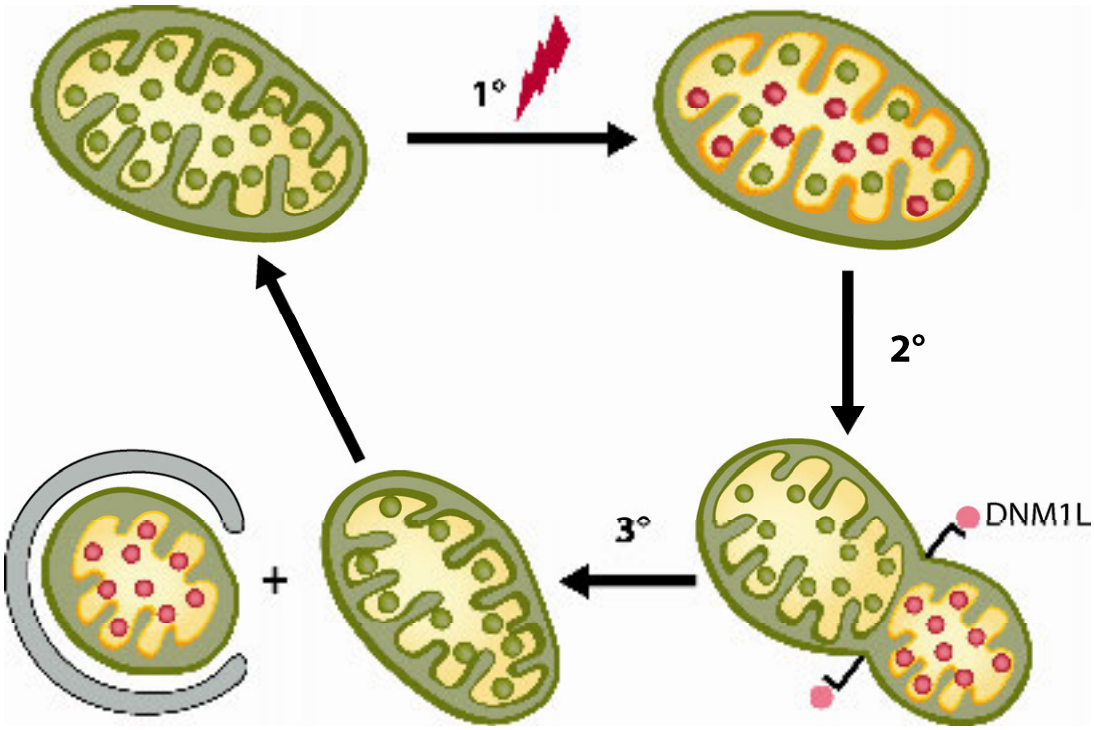

Figure 1 A model of mitochondrial fission. 1) Mitochondrion accumulates damage due to either external or internal factors. 2) Fission may start with compartmentalization of accumulated damage. The inner membrane forms two different compartments. Dynamin 1 like –protein (DNM1L) attaches to a receptor and marks the site of division. 3) The mitochondrion splits into two organelles: one healthy and one harbouring the damaged particles. This remnant can be processed further, either by mitophagy, which involves autophagosome (gray organelle) or by fission into the larger mitochondrial network (not shown).

Mitophagy, a quality control pathway of mitochondria, works closely with the help of mitochondrial dynamics [Reviewed in (Youle and Narendra, 2011)]. Mitophagy, or mitochondrial autophagy, is the process of catabolism of mitochondria. In mammals and yeast, mitophagy is preceded by mitochondrial fission to divide the mitochondria into manageable pieces for encapsulation (Twig et al., 2008). Mitophagy is also required to maintain steady state levels of mitochondria and adjusting the amount of mitochondria in the event of stress (Tal et al., 2007). Two important proteins in the process are PTEN-induced putative kinase protein 1 (PINK1) and parkin, an E3 ubiquitin ligase. The exact mechanism how PINK1 distinguishes healthy mitochondria from damaged is unknown. However, in cell culture experiments PINK1 acts as a recruiting factor (Figure 2). In normal conditions, PINK1 is constantly imported into mitochondria, via an unknown pathway. When the mitochondrial membrane potential is lost, PINK1 import is disrupted and PINK1 immediately accumulates on the mitochondrial outer membrane, to where parkin is recruited and bound (Narendra et al., 2010). Parkin ubiquitylates specific proteins on the outer membrane using a mechanism that resembles endoplasmic reticulum associated degradation (Heo et al., 2010; Xu et al., 2010). The proteins

participating in this event include MFN1, MFN2 and Miro, thus linking autophagy, MAM and dynamics (Chan et al., 2011; Wang et al., 2011).

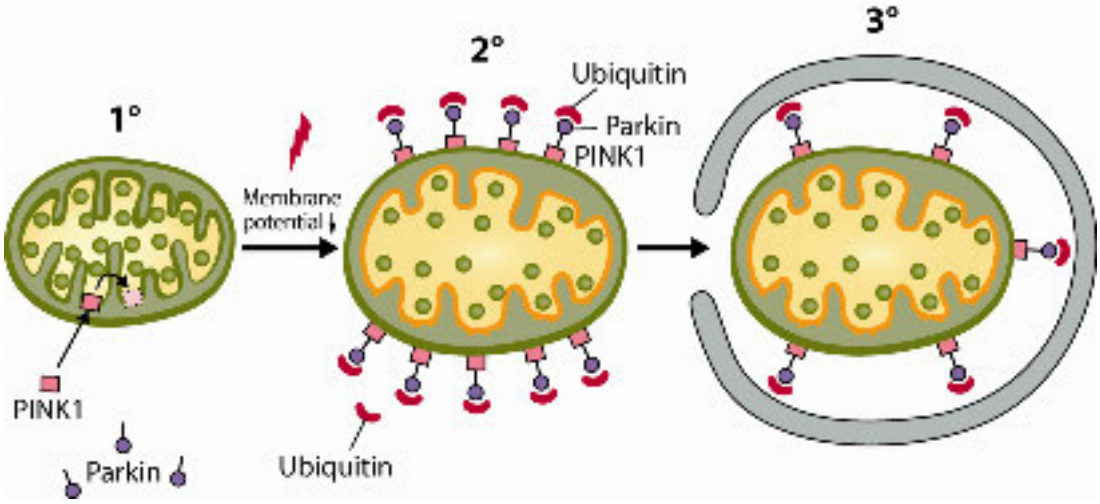

Figure 2 A model of PINK1/Parkin induced mitophagy. 1) In normal situation, PINK1 is continuously imported to inner membrane, where PINK1 is degraded quickly (light pink square = PINK1). 2) When mitochondrial membrane potential is lost, PINK1 import is disrupted and it starts to accumulate on the outer membrane, recruiting Parkin. PINK1/Parkin complex binds to ubiquitin. 3) PINK1/Parkin/Ubiquitin complex induces the autophagic elimination of mitochondria. Grey organelle: Autophagosome

Fusion and fission also participate in apoptosis: fusion protects the cell from apoptosis whereas fission has a positive role in it (Frank et al., 2001; Olichon et al., 2003). These functions strengthen the position that dynamics is a way to control the wellbeing of individual mitochondria and the whole network.

4.4 THE STRUCTURE OF A MITOCHONDRION

The human mitochondrion is a two-membrane-bound organelle, characterized by cristae formed by the inner membrane (IM) (Figure 3). The outer and inner membranes divide the organelle into the intermembrane space and the matrix. These areas provide the mitochondria with different compartments and environment to facilitate its functions. The inner and outer membranes are phospholipid bilayers with protein complexes embedded into them. The differences in the contents of the intermembrane space and matrix form the basis of the most important mitochondrial function, adenosine triphosphate (ATP) generation by the electron transport chain (ETC, also known as respiratory chain): an electrochemical gradient which is composed from the transport of H^+ ions from matrix to the intermembrane space through the electron transport chain, is used to transform nutrient energy in the conversion of ADP $+P_i$ (Adenosine

diphosphate and Inorganic Phosphate) to ATP when the H^+ ions flow back to the matrix through ATP synthase (Complex V).

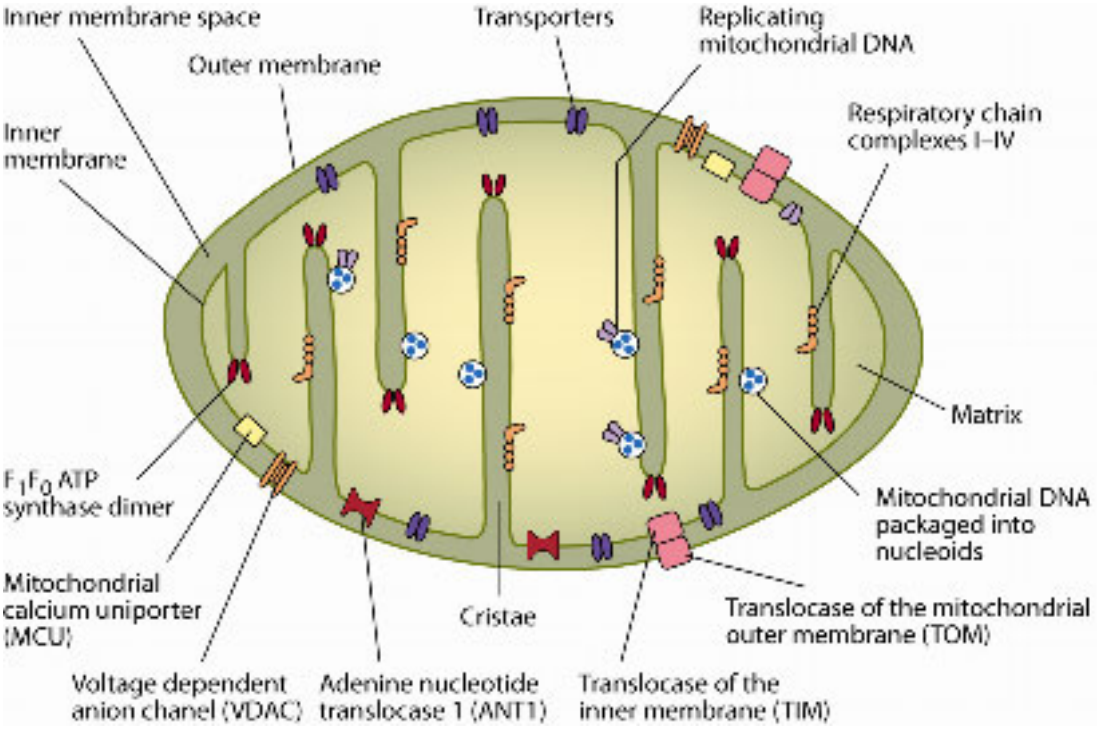

Figure 3 The structure of the human mitochondrion.

4.4.1 OUTER MEMBRANE

The mitochondrial outer membrane hosts a variety of integral membrane proteins, protein complexes and structures, which are involved in the transport of proteins from the cytosol to mitochondria (Figure 3) (Schein et al., 1976; Wurm et al., 2011). Molecules under 5 kDa are able to freely diffuse through membrane pores, whereas larger proteins need a transporter, such as translocase of the mitochondrial outer membrane (TOM) [For a review (Schleiff and Becker, 2011)]. It also acts as a signalling platform for apoptosis and innate antiviral immunity [(Seth et al., 2005) and reviewed in (Bogner et al., 2011)].

4.4.2 INTERMEMBRANE SPACE

The composition of the intermembrane space resembles the cytosol when smaller molecules (<5kDa) are considered (Figure 3). Larger molecules have to be transported and thus the composition of the intermembrane space does not resemble either cytosol or matrix. The intermembrane space also harbours cytochrome c, the transporter of electrons between complex III and

IV (CIII and CIV). In the case of apoptosis, cytochrome c is released from the intermembrane space to cytosol through BCL-2 controlled membrane permeabilisation [Reviewed in (Bogner et al., 2011)].

4.4.3 INNER MEMBRANE

The folds of the inner membrane form cristae, which increase the surface area of the membrane but also form different functional regions across the inner membrane (Figure 3). In *S. cerevisiae* the cristae are formed by F_1F_0 ATP synthase dimers, which deform the lipid bilayer of the inner membrane by forming a row of ATP synthases, which curve the bilayer into cristae (Davies et al., 2012). The inner boundary region is closely apposed to the outer membrane and thus functions in protein and lipid trafficking and respiratory chain complex assembly. Cristae project inside the matrix, house assembled respiratory complexes and act as a site for mitochondrial DNA (mtDNA) replication (Figures 3 and 6) (Brown et al., 2011; Vogel et al., 2006). Crista junctions, between the boundary region and cristae, connect these two structures together with tubular structure (Perkins et al., 1997).

4.4.4 MATRIX

The mitochondrial matrix contains necessary enzymes for the complete citric acid cycle. It also contains mtDNA, ribosomes and acts as the main Ca^{2+} storage site (Figure 3) (Rossi and Lehninger, 1964).

The storage of Ca^{2+} is not a passive process in mitochondria. Instead, mitochondria are able to rapidly import and export Ca^{2+}. The import of Ca^{2+} is mediated via mitochondrial Ca^{2+} uniporter (MCU) (Figure 3). The activity and biochemical properties of MCU have been characterized long ago, but the protein itself has remained elusive until recently [(Baughman et al., 2011; De Stefani et al., 2011) and for a review (Carafoli, 2003)]. The Ca^{2+} uptake system is exposed to microdomains of high [Ca^{2+}], in the vicinity of endoplasmic reticulum and plasma membrane Ca^{2+} channels, which explains the rapid reaction to cell stimuli (Rizzuto et al., 1993; Rizzuto et al., 1998). Matrix [Ca^{2+}] increases stimulate aerobic metabolism in mitochondria, as well as sensitizing mitochondria to apoptotic stimuli (Hajnoczky et al., 1995; Jouaville et al., 1999; Pinton et al., 2001). Ca^{2+} is exported from mitochondria to the cytosol via Na^{2+}/ Ca^{2+} and possibly H^+/Ca^{2+} exchangers (Jiang et al., 2009; Palty et al., 2010). In neurons, Ca^{2+} ions are required for neuronal survival, but too high concentrations lead to cell injury and death (Penn and Loewenstein, 1966; Simon et al., 1984).

4.5 NUCLEUS AND MITOCHONDRIA

Unlike other cellular organelles, mitochondria and nucleus have their own genomes, mtDNA and nuclear DNA (nDNA) (Nass and Nass, 1963). Mitochondria are not able to function independently, however, as the human mitochondrial genome encodes only 37 genes: 13 are subunits for respiratory complexes, 22 for mitochondrial transfer ribonucleic acids (RNA) and 2 for ribosomal RNAs (Anderson et al., 1981). The respiratory subunits form the most conserved part of the respiratory chain complexes (Yip et al., 2011). This is a tiny portion of the total ~1500 proteins that are currently thought to be imported and to take part directly in the function of mitochondria (Mootha et al., 2003; Pagliarini et al., 2008; Sickmann et al., 2003).

The relationship of mitochondria and nucleus is not biased, as mitochondria produce signals of the metabolic state, affecting the nuclear responses. Adenosine monophosphate (AMP)/adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD+/NADH) ratios, and Acetyl Coenzyme A (Acetyl-CoA) levels are sensed by AMP-activated protein kinase, sirtuin 1 and histone acetyltransferase KAT2A (GCN5), which act through peroxisome proliferator-activated receptor gamma coactivator 1-alpha, a transcription coactivator and ultimately affect the transcription of genes involved in energy metabolism (Canto et al., 2009; Canto et al., 2010; Jager et al., 2007; Lerin et al., 2006).

4.6 MITOCHONDRIAL DNA

The size and organization of mtDNA varies by species. In humans, mtDNA is circular, double-stranded DNA with a length of 16569 base pairs (bp). However, some plants such as muskmelon may have mitochondrial DNA as long as 2.8 Mb (Rodriguez-Moreno et al., 2011). The mtDNA is not always circular as some species also have linear organization (Grant and Chiang, 1980). Unlike nDNA, the copy number of mtDNA in human cells varies usually between 100-10000 copies. Usually, the mtDNA population is homoplasmic: just one mtDNA genotype exists in the organism (Figure 4-1). In heteroplasmy, two or more different mtDNA populations exist in the same cell (Figure 4-2). Heteroplasmy is important in the context of disease, as one of the mtDNA genotypes may harbour a disease causing mutation (Holt et al., 1990; Wallace et al., 1988). Homoplasmic mtDNA mutations are considered to be incompatible with life in general, but several descriptions of diseases caused by homoplasmic mtDNA mutations exist (Degoul et al., 1995; Gotz et al., 2012; Tulinius et al., 1995). Most likely, homoplasmic mutations are more common than previously thought.

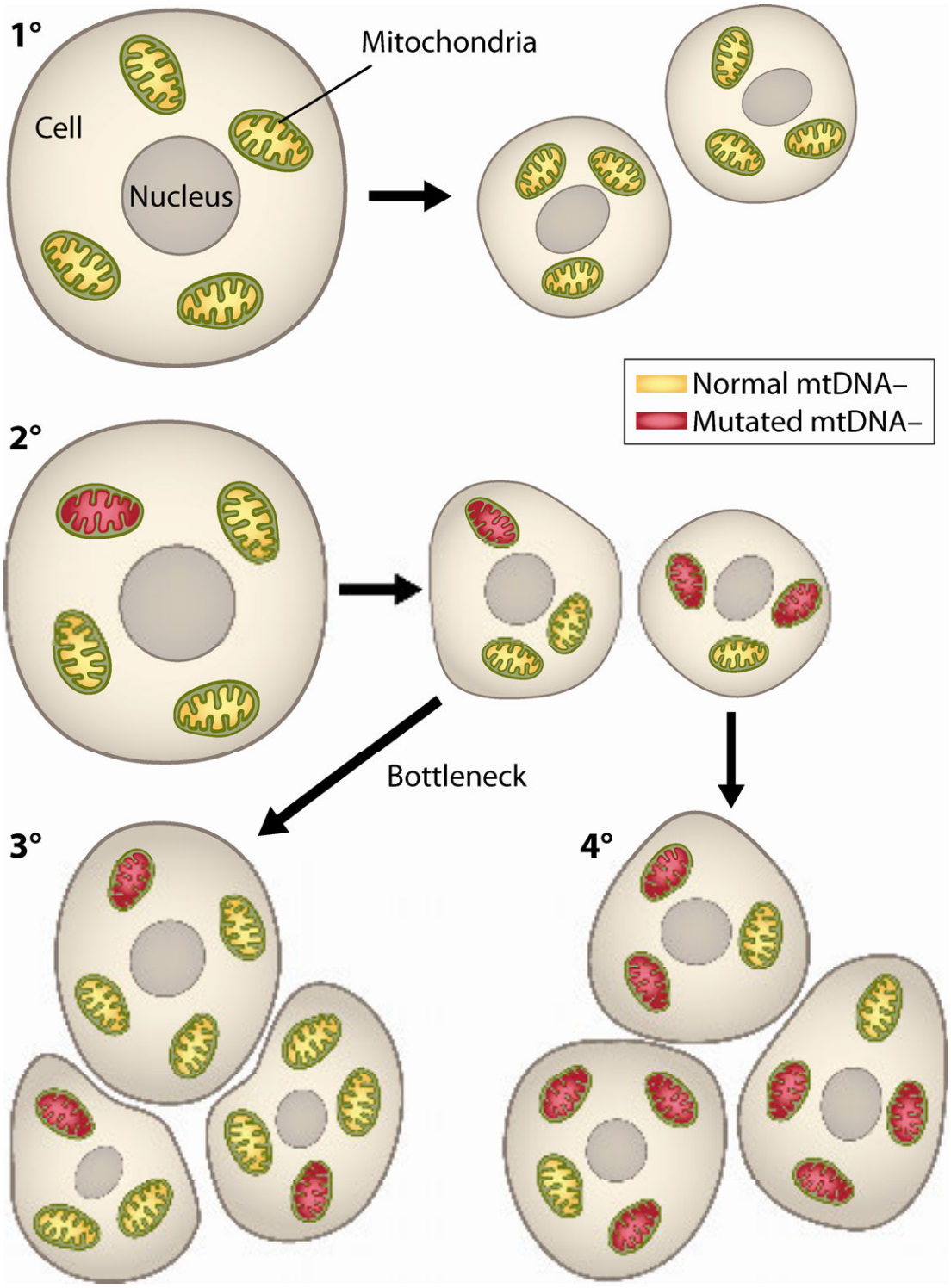

Figure 4 Mitochondrial genetics. mtDNA mitochondrial DNA. For simplicity, in this picture yellow mitochondria represent mitochondria with just one mtDNA population (1, Homoplasmy) and red mitochondria represent mitochondria with several mtDNA populations, of which at least one is mutated (2, Heteroplasmy). 1) During oocyte maturation, the copy number of mtDNA per cell gets lower and daughter cells have lower copy number of mtDNA. This is called the bottleneck effect. 2) During oocyte maturation, the mtDNA copies are randomly distributed. 3) and 4) During the development of the oocyte, mtDNA copies are randomly replicated. However, mitochondria are able to withstand a certain amount of heteroplasmy (3). When the threshold for disease is passed (4), clinical symptoms start to develop. The threshold is specific for each tissue and mtDNA mutation.

Mitochondrial DNA is exclusively maternally inherited. In fact, paternal mitochondria are actively degraded during fertilization (Al Rawi et al., 2011; Sato and Sato, 2011). When a fertilized oocyte starts dividing, the mtDNA copy number per cell drops dramatically. This has several consequences: if mtDNA population is heteroplasmic, the random replication of mtDNA molecules can lead to increased ratio of mutated mtDNA molecules (Figure 4-3 and 4-4) (Wai et al., 2008). This is called the genetic bottleneck. The second consequence is that when the amount of mutated mtDNA reaches a certain level, called threshold, disease and its symptoms develop (Figure 4-4) (Miyabayashi et al., 1992; Shoffner et al., 1990; Wallace, 1986). This also means that mitochondria are able to withstand a certain amount of mtDNA damage or mutated mtDNA molecules. Organisms have also a strong evolutionary pressure for mtDNA homoplasmy and against deleterious mtDNA mutations, especially in the protein coding regions(Fan et al., 2008; Stewart et al., 2008).

4.7 ORGANIZATION OF MITOCHONDRIAL NUCLEOIDS

Mitochondrial DNA is organized in nucleoids, which are divided evenly throughout the mitochondrial matrix (Satoh and Kuroiwa, 1991). One nucleoid has 1-10 mtDNA molecules attached, in addition to proteins and RNA (Brown et al., 2011; Kukat et al., 2011). In mammals, the most abundant nucleoid protein is mitochondrial transcription factor A (TFAM) (Kaufman et al., 2007; Larsson et al., 1998). One mtDNA molecule has approximately 450 binding sites for TFAM and it binds mtDNA efficiently with two high-mobility group boxes (Fisher et al., 1992; Kaufman et al., 2007). In addition, it also functions as a transcription factor and is able to bind mtDNA strongly at promoters. TFAM is a strong regulator of mtDNA copy number, a dramatic decrease in mtDNA copy number is observed in TFAM knock-out mice and results in embryonic lethality whereas overexpression of TFAM leads to increased mtDNA copy number (Ekstrand et al., 2004). LON protease regulates the TFAM protein level in *Drosophila Melanogaster* (*D. Melanogaster*) cells and thus mtDNA copy number (Matsushima et al., 2010).

The structure of mitochondrial nucleoids has been recently studied using superresolution microscopy (Brown et al., 2011; Kukat et al., 2011). With superresolution microscopy, resolutions approaching 20-50 nm in the lateral dimension are achievable, thus making these techniques able to recognize structures inside the matrix, between cristae (average span between cristae is ~68 nm). The studies were performed using several different cell lines: human primary fibroblasts, several human cancer cell lines, mouse fibroblasts and kidney cells from two different organisms. There were 1-6

copies of mtDNA per nucleoid, according to both studies. Both published studies arrived at different conclusions about the size and shape of the mitochondrial nucleoids: Kukat and co-workers report uniform diameter and size, ~100 nm, per nucleoid whereas Brown and co-workers state a large variation in the size as within one standard deviation there was 25-fold variation in the volume. In addition, Brown and co-workers report that the nucleoids had multiple shapes, ellipsoidal nucleoids being the most common (35%). Brown and co-workers conclude that the large variation in ellipsoidal nucleoid volume is strongly influenced by the genome content of nucleoid. Other shapes suggested interaction and binding with the inner membrane. For an ellipsoidal shape, they calculated that on average the TFAM/mtDNA ratio is enough for TFAM to cover all mtDNA molecules in a cell. For other shapes, this is not the case and it suggests that other nucleoid proteins, currently unknown, stabilize mtDNA in other nucleoid conformations. A plethora of different proteins are associated with the nucleoid complex, such as ribosomal proteins, chaperones, quality control proteins, helicase and RNA binding proteins.

In human cells, nucleoids seem to function in connection with the actin network, thus connecting the mitochondrial DNA and nucleoids to a cellular support structure and suggesting they may take part in the movement of mitochondria (Iborra et al., 2004; Reyes et al., 2011). In yeast, the replisome locates to a membrane spanning protein complex (Boldogh et al., 2003; Meeusen and Nunnari, 2003). This complex spans both inner and outer membranes and links to the actin network outside mitochondria. The minimal two-membrane structure is composed of Mip1 (DNA polymerase, homolog of POLG1), Mmm1 (Outer membrane protein) and Mgm101 (DNA binding protein). The two-membrane structure may function as a replication factory, mtDNA position stabilizer or something else. Although the two-membrane structure has been found in *S. cerevisiae*, no mammalian or human equivalent has been found so far. Other strong differences between mammalian and yeast mtDNA replication exist, such as lack of the DNA polymerase gamma accessory subunit and as such, the two-membrane structure might be a yeast specific feature (Lucas et al., 2004).

4.8 MITOCHONDRIAL REPLISOME

The minimal mitochondrial replisome is composed of POLG, TWINKLE and mtSSB *in vitro*, as they are able to replicate mtDNA sized and pre-primed circular templates (Korhonen et al., 2004). However, based on the current models of mtDNA replication, these few proteins are clearly not enough to replicate mtDNA *in vivo*: mitochondrial RNA polymerase (POLRMT) is required to transcribe mtDNA and thus produce an RNA primer for the initiation of leading strand synthesis and possibly also for the lagging strand.

TFAM and mitochondrial transcription factor 2B (TFB2M) are required for the initiation of transcription. Although transcription and replication of mtDNA are strongly linked, it is not known how these processes work together. Further, an *in vitro* model for transcription and replication of mtDNA working in tandem has not been produced yet (Fuste et al., 2010).

4.8.1 DNA POLYMERASE GAMMA

The mitochondrial DNA polymerase, DNA polymerase gamma, is the only known DNA polymerase that functions in mitochondria. In humans, DNA polymerase gamma is a holoenzyme, consisting of one catalytic core (POLG1) and two accessory subunits (POLG2) (Gray and Wong, 1992; Yakubovskaya et al., 2005). The catalytic core is a 140 kDa protein encoded by *POLG1* (Bolden et al., 1977; Lecrenier et al., 1997). The catalytic core consists of 3'-5' exonuclease and polymerase domains, which are separated by a spacer region. Both exonuclease and polymerase domains have in total six highly conserved sequence motifs: Exo I, II and III for the exonuclease domain and Pol A, B and C for the polymerase domain (Figure 5). In the spacer region, four conserved sequence blocks can be identified: γ1, γ2, γ3 and γ4 (Figure 5) (Luo and Kaguni, 2005).

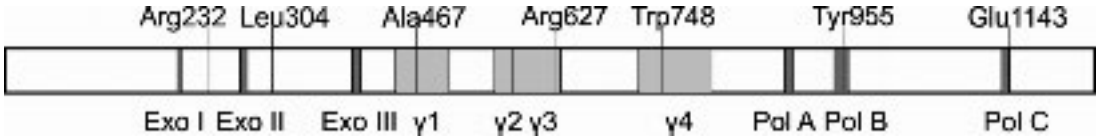

Figure 5 Linear structure of human POLG1. Exo I, II and III: conserved sequence motifs of exonuclease domain. γ1, γ2, γ3 and γ4: conserved sequence blocks of exonuclease domain. Pol A, B and C: Conserved sequence motifs of polymerase domain.

The accessory subunit is a 55 kDa protein, encoded by *POLG2.* It also has three conserved domains (Fan et al., 2006). Although it mainly functions in increasing processivity of the holoenzyme, accessory subunit is able to bind double stranded DNA (dsDNA) alone (Carrodeguas et al., 2002; Fan et al., 2006; Lim et al., 1999). POLG2 is required for the function of the mtDNA replisome and it takes part in determining the mtDNA content of mitochondrial nucleoids (Di Re et al., 2009; Farge et al., 2007).

The catalytic core is able to polymerize and bind DNA alone, without the help of the accessory subunits (Gray and Wong, 1992; Wernette and Kaguni, 1986). However, the overall processivity of the enzyme is raised as much as 100-fold with the help of accessory subunits (Fan et al., 2006; Johnson et al., 2000; Lim et al., 1999). In addition, the fidelity of the enzyme is increased with the accessory subunit. The catalytic subunit also has 5′-deoxyribose phosphate (dRP) lyase activity, which works in DNA repair by removing the

dRP moiety, thus making the single nucleotide gap filling possible (Longley et al., 1998b).

DNA polymerase gamma is adaptable in the conditions required for DNA polymerase activity: broad pH range (7.5-9.5), ability to use various primer-templates and moderate to high salt concentration. POLG does require a divalent cation and has high affinity for deoxyribonucleotides (dNTP) and is able to achieve the highest polymerase activity with high primer-density natural sequence DNA, such as gapped double stranded calf thymus DNA, which is routinely used in the DNA polymerase assays (Wernette and Kaguni, 1986). POLG is also able to utilize natural sequence RNA as a template, although not as effectively as natural sequence DNA (Murakami et al., 2003). On singly-primed, natural sequence M13 DNA, the human catalytic core has processivity of 50 nt, but the reconstituted holoenzyme is able to produce products up to 7 kb (Johnson et al., 2000; Lim et al., 1999; Longley et al., 1998a). The catalytic core is able to replicate short stretches of DNA alone, without the accessory subunit. This may have relevance in mtDNA repair, in which POLG1 is known to participate and might act without the accessory subunit (Mitra et al., 2007; Pinz and Bogenhagen, 2005).

POLG is a member of the family A DNA polymerase group. The basic structure of the POLG catalytic core (right-hand configuration with thumb, palm and fingers) is very close to other members of the Pol I family, but the spacer makes it unique (Lee et al., 2009). The spacer domain can be divided into intrinsic processivity (IP) and accessory interacting determinant (AID) subdomains (Lee et al., 2009). The IP subdomain has active residues, which bind upstream of the primer-template DNA (ptDNA) and explain the high intrinsic polymerase activity of the catalytic core alone, compared to other Pol I family polymerases. Whereas the IP subdomain takes part directly in the polymerase activity, the AID subdomain forms an important interface with POLG2.

The POLG holoenzyme is a heterotrimer in mammals (Gray and Wong, 1992; Olson et al., 1995; Yakubovskaya et al., 2005). The two accessory subunits interact differently with the catalytic core, with one being the proximal and the other the distal subunit (Lee et al., 2009; Lee et al., 2010b). The proximal subunit has a plethora of interactions with the catalytic core, both hydrophilic and hydrophobic. Deletion and mutation of the hydrophobic residues result in almost complete lack of subunit interaction and diminish the polymerase activity of the holoenzyme to the same level as catalytic core alone (Fan et al., 2006). The distal subunit has only a direct interaction with the catalytic core through only one salt bridge and one van der Waals interaction. In *Drosophila melanogaster*, the POLG holoenzyme is indeed able to function with a monomeric accessory subunit (Wernette and Kaguni, 1986). The role of the distal accessory subunit is to increase the

polymerization rate of the POLG holoenzyme, whereas the proximal accessory subunit increases the DNA binding affinity of the holoenzyme (Lee et al., 2010a; Lee et al., 2010b). This increase does not come without a cost, as POLG2 stimulation of the POLG1 DNA synthesis is partly through diminishing the exonuclease activity of the holoenzyme (Farge et al., 2007; Johnson and Johnson, 2001a). The accessory subunit and the spacer region are features of metazoans. For example, *S. cerevisiae* does not have either (Foury, 1989; Ropp and Copeland, 1996).

Human POLG holoenzyme has high fidelity, with just one error per 3 x 10^5 base pairs, compared for example to the rat Pol β, which has an error rate of one error per 1600-51000 base pairs (Ahn et al., 1997; Johnson and Johnson, 2001b; Kaguni and Olson, 1989). This high fidelity is due to exonucleatic proofreading activity of the holoenzyme and high nucleotide selectivity (Johnson and Johnson, 2001a; Johnson and Johnson, 2001b; Longley et al., 2001). The error rate is modified by several factors: nucleotide pool imbalances, abasic sites and oxidative damage on the template DNA (Kunkel and Soni, 1988; Pinz and Bogenhagen, 1998; Wernette and Kaguni, 1986). This is clinically relevant, as for example, mutations in *ANT1* (adenine nucleotide translocator 1) and *RRM2B* (ribonucleotide reductase M2 B) mutations cause progressive external ophthalmoplegia (PEO), a syndrome characterized with multiple mtDNA deletions, and *TYMP* (a gene involved in the nucleotide salvage pathway) mutations lead to mtDNA depletion (Kaukonen et al., 2000; Nishino et al., 1999; Tyynismaa et al., 2009).

The high fidelity of the POLG holoenzyme is partly achieved due to the 3'-5' exonuclease activity of the holoenzyme. The exonuclease activity of POLG requires the same broad conditions as polymerase activity and seems to have a clear preference for dsDNA containing 3'-terminal mispairs (Kaguni and Olson, 1989; Kunkel and Mosbaugh, 1989; Kunkel and Soni, 1988). POLG exonuclease mispair specificity is high and the cost of proofreading during DNA synthesis low (Kaguni and Olson, 1989; Longley and Mosbaugh, 1991). The ability to transfer a mispaired 3'-terminus of the DNA strand from pol to the exonuclease domain and the 10-fold higher dissociation from the pol active site, in the case of mispaired rather than correctly paired 3'-terminus, result in 20 to 200 fold increase in fidelity of the enzyme (Johnson and Johnson, 2001a).

4.8.2 HELICASE TWINKLE

Human mitochondrial DNA helicase, TWINKLE, which is encoded by *C10ORF2* was discovered in 2001 (In this thesis, *Twinkle* is used as synonym for *C10ORF2*) (Spelbrink et al., 2001). Similar to other components of mitochondrial DNA replication machinery, it resembles the T7 gene product

4 primase-helicase (T7 GP4). The highest homology between human TWINKLE and T7 GP4 is in the helicase domain, which supports the fact that the human TWINKLE does not have any primase activity (Farge et al., 2008; Spelbrink et al., 2001). In addition, bioinformatic analysis of mammalian TWINKLE sequences show that it has lost the required primase domains for actual synthesizing the short RNA primers (Shutt and Gray, 2006). The size of TWINKLE protein is 684 amino acids. A splice variant named TWINKY, which lacks the amino acids 579-684, was found together with TWINKLE, but the function and relevance of TWINKY is still unknown (Spelbrink et al., 2001). TWINKLE, as other replicative helicases, forms a hexameric ring (Farge et al., 2008; Ziebarth et al., 2007; Ziebarth et al., 2010). However, it does also form a heptameric ring routinely. This structure is not limited to TWINKLE, as some other helicases, such as *Methanobacterium thermoautotrophicum* minichromosomal maintenance protein also form this structure, however the function or relevance is again unknown (Yu et al., 2002; Ziebarth et al., 2010). TWINKY, however, is unable to form oligomeric structures with itself or TWINKLE. Recombinant TWINKLE has strong ATPase activity and it unwinds DNA in 5' to 3' direction (Korhonen et al., 2003).

Human recombinant TWINKLE is able to bind circular single stranded DNA (ssDNA) (Jemt et al., 2011). After the hexameric ring has bound circular ssDNA, TWINKLE is able to stay bound longer than on linear ssDNA. TWINKLE is also able to start unwinding ssDNA with an oligomer primer on its own. Together with POLG, they are able to replicate through an artificial dsDNA bubble (resembling human mtDNA D-loop structure) (Jemt et al., 2011). Mitochondrial single-stranded DNA binding protein (mtSSB) is able to stimulate the helicase activity of TWINKLE, but it does not inhibit the unwinding of dsDNA, when the template is coated with mtSSB (Jemt et al., 2011). Taken together, these results show that TWINKLE is able to function on a mtDNA-like template, together with other proteins of the replisome.

4.8.3 MITOCHONDRIAL SINGLE-STRANDED DNA BINDING PROTEIN

Unlike mitochondrial helicase and DNA polymerase, the mtSSB does not share homology with its T7 counterpart. MtSSB shares 50% similarity with *Escherichia coli* single stranded binding protein (*E.coli* SSB) deduced amino acid sequence and its tertiary structure and functionality resembles *E. coli* SSB closely (Tiranti et al., 1993; Webster et al., 1997; Yang et al., 1997). Human mtSSB is a homotetramer; it binds ssDNA to protect ssDNA against damage and to coordinate the function of other proteins of the replisome (Korhonen et al., 2004; Yang et al., 1997). MtSSB has been shown to increase the activities of the other proteins in the replisome: POLG and TWINKLE, and the overall function of the replisome (Korhonen et al., 2004). Knock-

down or knock-out of mtSSB in both human cells and *D. melanogaster* results in reduced mtDNA copy number (Farr et al., 2004; Farr et al., 1999; Maier et al., 2001; Oliveira and Kaguni, 2010; Ruhanen et al., 2010).

4.8.4 MITOCHONDRIAL RNA POLYMERASE

POLRMT and transcription factors (TFAM, TFB2M) form the basal mitochondrial transcription machinery (Falkenberg et al., 2002; Litonin et al., 2010). In addition to transcription, POLRMT acts as the mitochondrial primase (Chang and Clayton, 1985; Chang et al., 1985; Fuste et al., 2010; Wanrooij et al., 2008). POLRMT has been proposed to generate the RNA primers required for the initiation of both leading and lagging strand synthesis. The synthesis of the leading strand (heavy strand) is primed by an RNA primer generated by POLRMT from the transcription initiation from light strand promoter (LSP) (Chang and Clayton, 1985). This primer is extended by POLG, but the majority of initiation events are terminated inside the D-loop, about 600 bp from the LSP promoter. These terminated strands stay hybridized to the parental mtDNA molecule, thus forming a triplex structure, the D-loop (Arnberg et al., 1971; ter Schegget et al., 1971).

The initiation of lagging strand DNA synthesis requires that the leading strand synthesis has advanced about 2/3 of the mtDNA, exposing a non-coding DNA, which contains the origin of lagging strand (Kang et al., 1997; Martens and Clayton, 1979). This exposed non-coding region takes the shape of a stem-loop, from which POLRMT is proposed to synthesize the RNA primer in a sequence and secondary structure dependant manner (Fuste et al., 2010; Wanrooij et al., 2008). As the mtDNA replication model is a highly debatable subject, it can be summarized that the full role of POLRMT in mtDNA initiation is still under debate.

4.9 REPLICATION OF MTDNA

The replication of mtDNA was first described to be strand-asynchronous, with strand-specific origins of replication (Figure 6-1)(Berk and Clayton, 1974). This model was challenged by results achieved by using two-dimensional agarose gel electrophoresis. Two-dimensional agarose gel electrophoresis is an established method, which is used to study replication intermediates and the replication fork. The two-dimensional agarose gel electrophoresis results supported an alternative explanation for mtDNA replication as having the following features: bidirectional, strand-coupled replication, resembling dsDNA replication seen in the nucleus (Figure 6-2) (Bowmaker et al., 2003; Holt et al., 2000; Yang et al., 2002). Instead of two

origins of replication, there would be an initiation zone, approximately 4 kb in size, from which the replication of both strands would begin.

The controversial bidirectional, strand-coupled mtDNA replication model was refuted with atomic force microscopy. Brown and co-workers did not find any reasonable proof to support strand-coupled DNA replication, such as Cairns structures or dsDNA replication forks (Brown et al., 2005). They did find, however, gapped circle structures, which are incompatible with strand-coupled DNA synthesis, but required for strand-asynchronous DNA replication. They found also evidence for possible alternative light-strand origins, but the relevance of these remains to be elucidated. Later, the strand-coupled mtDNA replication model was refined when it was found that the lagging strand is initially laid down as RNA, before being converted to DNA (Figure 6-3) (Yang et al., 2002; Yasukawa et al., 2006; Yasukawa et al., 2005). This new model was named ribonucleotide incorporation throughout the lagging strand (RITOLS). Further studies have confirmed the existence of stable, long RNA intermediates on the lagging strand (Brown et al., 2008). The role of these intermediates is unclear: they may be processed to form RNA primers for the lagging strand, but some studies have not found proof to confirm this (Brown et al., 2008).

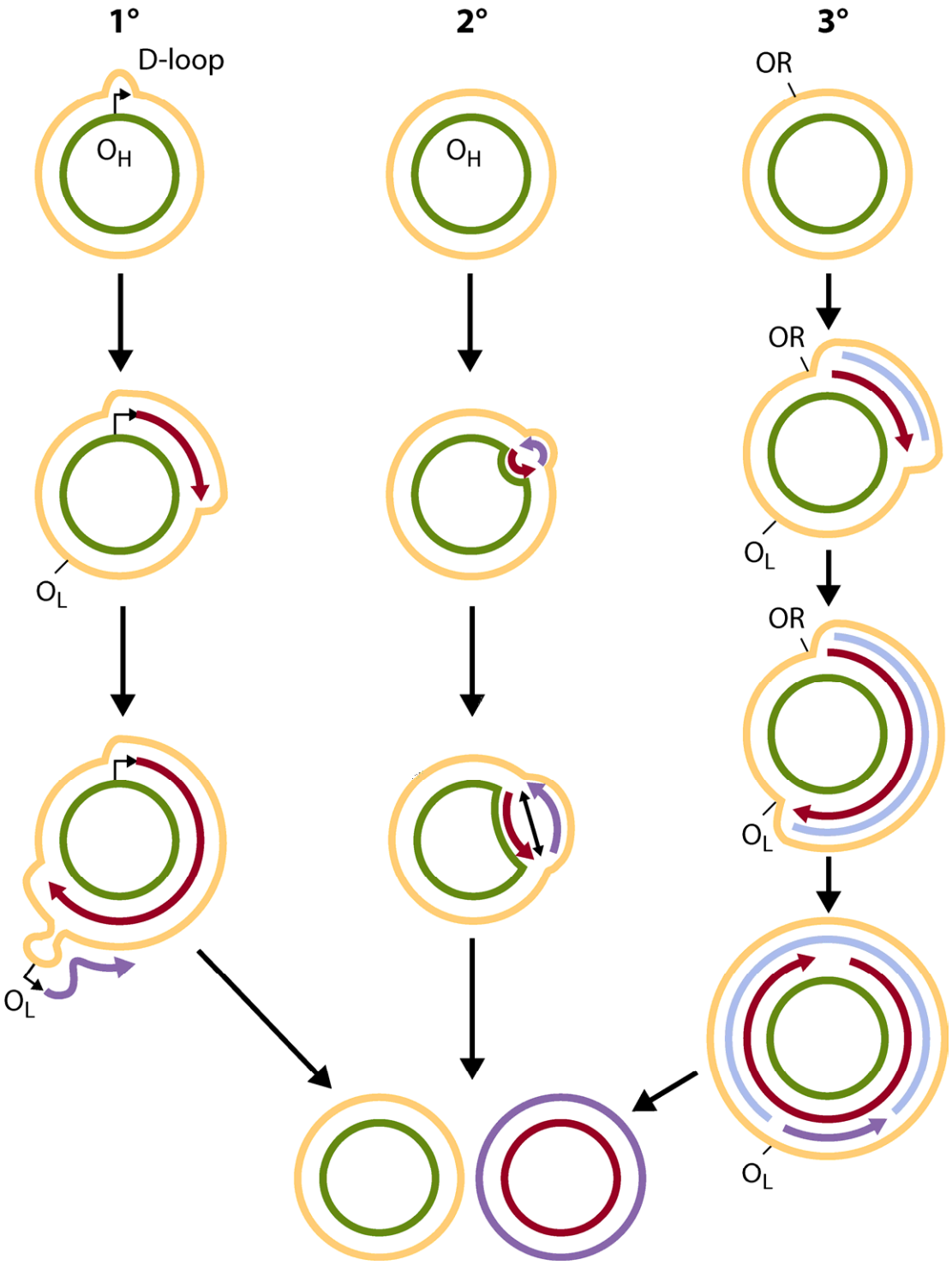
1°
D-loop
O_H
O_L
2°
O_H
3°
OR
OR
O_L
OR
O_L
O_L
O_L

Figure 6 Models of mitochondrial DNA replication. Yellow: light (lagging) strand. Green: heavy (leading) strand. Burgundy: replicating heavy strand. Purple: replicating light strand. Light blue: Newly synthesized RNA strand O_H: origin of heavy strand. O_L: origin of light strand. OR: origin of RITOLS leading strand. 1) Strand- asynchronous model of mitochondrial DNA replication. The replication begins from the O_H from RNA primer made by mitochondrial RNA polymerase. When the replication of heavy strand reaches O_L , it forms a stem-loop, to which mitochondrial RNA polymerase synthesis the RNA primer for the light strand and from the replication of the light strand begins. 2) Bidirectional, strand-coupled mtDNA replication model. The replication of both strands begins from multiple zones, downstream from O_H and continues bidirectionally. 3) Ribonucleotide incorporation throughout the lagging strand (RITOLS) model of mitochondrial DNA replication. The replication of heavy strand begins from discrete origin, OR and continues unidirectionally. At the same time, the displaced lagging strand is initially laid down as RNA. When the heavy strand replication reaches O_L , the new RNA copy of lagging strand is replaced by DNA strand.

Although the controversy is still far from over, we can conclude that since 2008, no major challenges have been presented to the current RITOLS model of mtDNA replication. The RITOLS model resembles the original strand-asynchronous model as transcription of the light strand provides the RNA primer required for the initiation of mtDNA replication at the heavy strand origin (Figure 6-3). The heavy strand, or leading strand, is replicated about two thirds and concomitantly covered by RNA, until the replication fork arrives at the light strand origin. The ssDNA forms a stem-loop, from which POLRMT synthesizes an RNA primer. On the light, or lagging strand, DNA maturation begins from this primer: the RNA primers are removed with the help of ribonuclease 1, DNA replication helicase 2 homolog and flap structure-specific endonuclease 1, and the gap is filled by POLG.

4.10 REPAIR OF MTDNA

The 10-50 fold higher rate of mutation accumulation in mtDNA than in nDNA and higher levels of oxidative damage to mtDNA puts mtDNA repair mechanisms in focus; indeed, some time ago the whole concept of mtDNA repair was questionable, but now current research has confirmed that most of DNA repair pathways exist also in mitochondria.

4.10.1 SOURCES OF MTDNA DAMAGE

The most important cause of mtDNA damage is intrinsic factors, such as reactive species, which are produced during the normal metabolism of the mitochondria and due to external factors. Both ROS and nitrogen species (RNS) are found inside mitochondria. ROS are produced during oxidative phosphorylation as electrons are transported through the ETC, with the final destination of an oxygen molecule. However, 0.2% of electrons are transported to oxygen prematurely from complexes I and III, leading to

formation of superoxide radicals, which can be converted to hydrogen peroxide [Reviewed in (Murphy, 2009)]. ROS can damage mtDNA and lead to formation of modified bases, sugar break down products, base-free sites and strand breaks [Reviewed in (Cooke et al., 2003)].

Peroxynitrite, a RNS, is produced from nitric oxide and superoxide anion. Peroxynitrite is able to oxidize DNA bases and fragment sugar, thus leading to strand breaks (Burney et al., 1999a; Burney et al., 1999b). A second important RNS is nitrous anhydride, which can deaminate primary amines of DNA bases and produce abasic sites (Burney et al., 1999b).

During the replication of mtDNA, POLG may also incorporate oxidized bases, thus leading to mtDNA damage. Mitochondria have various defense mechanisms against ROS and other stressors, such as antioxidants. In addition, specific enzymes that degrade oxidized nucleotides in the dNTP pool exist (Fujikawa et al., 1999). Furthermore, POLG has a 3'-5' exonuclease function that proofreads the newly synthesized DNA strand.

4.10.2 MECHANISMS OF MTDNA REPAIR

4.10.2.1 Base excision repair

The base excision repair (BER) pathway is the best characterized repair pathway in mitochondria. Both short- and long-patch BER (Single nucleotide and 2-6 nucleotides, respectively) exist in mammalian mitochondria (Akbari et al., 2008; Liu et al., 2008; Stierum et al., 1999b; Szczesny et al., 2008; Zheng et al., 2008). Short patch base excision repair is important in removing single lesions, whereas long-patch base excision repair is part of mtDNA replication in removing the RNA primers (Zheng et al., 2008).

In both short patch and long-patch base excision repair, the lesion such as 8-hydroxyguanine (the major oxidative lesion in mitochondria) is first removed by a DNA glycosylase. The remaining abasic nucleotide or nucleotides are then removed by AP endonuclease and the gap is filled by POLG (Mitra et al., 2007; Pinz and Bogenhagen, 1998). Then, the remaining displaced dRP (short patch base excision repair) or nucleotide flap (long-patch base excision repair) is cleaved. POLG is able to remove a single dRP from short patch base excision repair. In comparison to short patch base excision repair, in long-patch base excision repair the ssDNA flap is removed by DNA2 (nuclease/helicase) and FEN1. DNA2 cleaves ssDNA internally and finally FEN1 cleaves ssDNA in the junction of ssDNA and dsDNA (Liu et al., 2008; Zheng et al., 2008). While removing RNA primers the substrate is first cleaved by RNaseH1, and then filled by POLG, and the ssDNA flap is

removed by DNA2 and FEN1 (Cerritelli et al., 2003). Finally, the newly synthesized DNA patch is joined to the downstream sequence by DNA Ligase 3 (Lakshmipathy and Campbell, 1999b).

The removal of damaged nucleotides via DNA glycosylase and lyase is damage-specific. For example, 8-hydroxyguanine is removed by 8-Oxoguanine glycosylase, uracil is removed by uracil-DNA glycosylase, and thymine glycol is removed by endonuclease III family glycosylases (Dobson et al., 2000; Nilsen et al., 1997; Nishioka et al., 1999; Stierum et al., 1999a). Removal of an abasic nucleotide is catalyzed by APE1 or APE2, which both exist in the nucleus and mitochondria (Mitra et al., 2007; Tsuchimoto et al., 2001).

4.10.2.2 Mismatch repair

In mismatch DNA damage, a wrong nucleotide is inserted into the newly synthesized DNA strand. The presence of the mismatch repair pathway in mitochondria has been confirmed in two independent studies, first using rat liver mitochondrial extracts and then human cells (de Souza-Pinto et al., 2009; Mason et al., 2003). It was also established in these studies that the classical nuclear mismatch repair proteins do not locate to mitochondria. It seems that the mismatch binding activity is due to Y-box binding protein YB-1. However, the exact characteristics of this pathway are still unknown.

4.10.2.3 Double-strand break repair

As in other mechanisms of mtDNA damage repair, the knowledge of double-strand break repair is limited. We know from patients that recombination intermediates have been identified (Kajander et al., 2001; Kraytsberg et al., 2004; Poulton et al., 1993). Also, in *in vitro* studies, it has been shown that mammalian mitochondrial extracts have activity for homologous recombination and end-joining activities (Coffey et al., 1999; Lakshmipathy and Campbell, 1999a). Heteroplasmic mice with inducible expression of restriction endonucleases, to create double-strand breaks in mtDNA, did not have detectable amounts of mtDNA double stranded breaks. Instead, they had mtDNA molecules with deletions, thus confirming existence of double-strand break repair activity in mammalian mitochondria (Bacman et al., 2009; Fukui and Moraes, 2009; Srivastava and Moraes, 2005). The efficiency of double-strand break repair or possible lack of it is under debate, but the evidence for double-strand break repair in mammalian mitochondria is strong (Alexeyev et al., 2008; Kukat et al., 2008).

4.10.2.4 Nucleotide excision repair

Nucleotide excision repair requires removal of short stretches of damaged DNA, which are then refilled. The existence of nucleotide excision repair in mitochondria has not been confirmed and classical nucleotide excision repair substrates are repaired by alternative mechanisms in yeast (Yasuhira and Yasui, 2000).

4.11 FUNCTIONS OF MITOCHONDRIA

4.11.1 OXIDATIVE PHOSPHORYLATION

Oxidative phosphorylation (OXPHOS) produces ATP from the energy released by the oxidation of nutrients (Figure 7). OXPHOS is the most conserved function of mitochondria (Yip et al., 2011). Besides being used as energy, ADP/ATP also act as signalling molecules in the cell, thus making mitochondria signalling centres. The molecular machine required for OXPHOS, also known as respiratory chain or electron transport chain, residues in the inner membrane of mitochondria and consists of five different complexes.

4.11.1.1 Complex I

Complex I (also referred to as NADH dehydrogenase) is the largest of the subcomplexes. CI is constructed from 45 subunits and the total molecular mass is ~1000 kDa. The core of CI is highly conserved and is composed of 14 subunits, of which seven are encoded by mtDNA and seven are nuclear encoded (Yip et al., 2011). All of the other CI subunits are also nuclear encoded. The energy released by the tricarboxylic acid cycle (TCA, also known as citric acid cycle) is stored as NADH, which CI is able to use as a substrate (Figure 7). Recent publications of the CI crystal structure from *Escherichia coli*, *Thermus thermophilus* and *Yarrowia lipolytica* have helped to elucidate the mechanism of action of CI (Berrisford and Sazanov, 2009; Efremov et al., 2010; Efremov and Sazanov, 2011; Hunte et al., 2010; Sazanov and Hinchliffe, 2006). The structure of CI in these organisms can be roughly divided into membrane and peripheral arms, which form an L-shaped cluster. The function of CI can be simplified by using the structural data: at the top of L, NADH is oxidized and flavin mononucleotide acts as the electron acceptor. The released electrons are transported quickly via seven iron-sulphur clusters, which form a descriptive electric cord throughout the structure, to the quinone binding site, where ubiquinone is reduced to

ubiquinol. CI also transports protons from the matrix to intermembrane space: the proton transfer is done at the base of CI in the membrane arm via antiporters which translocate the protons by a structural change in the membrane arm. This change can be oversimplified to resemble the connected moving wheels of a locomotive engine. CI is also a prolific producer of ROS (Galkin and Brandt, 2005; Kussmaul and Hirst, 2006).

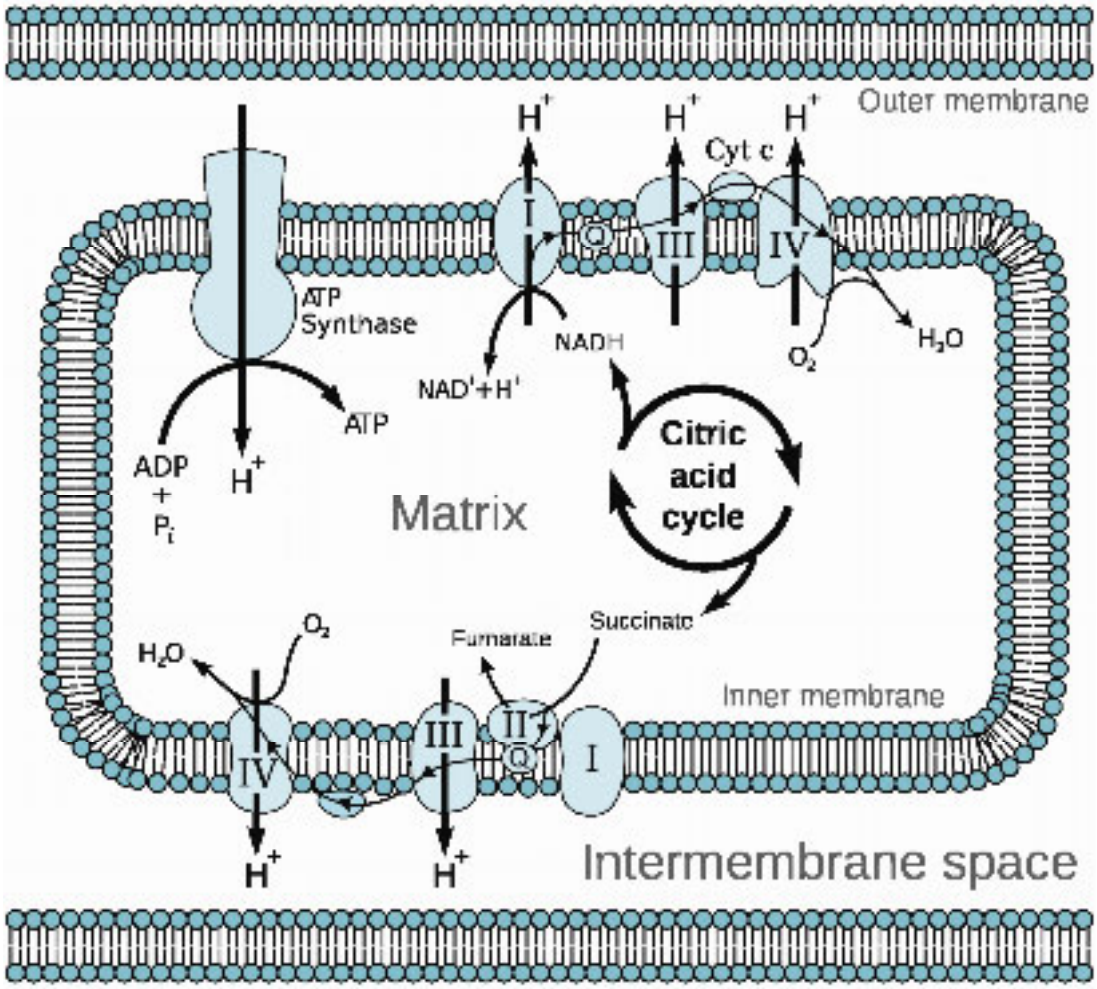

Figure 7 Schematic representation of oxidative phosphorylation. I-IV, complexes I-IV. Upper part: Complex I acts as the main electron entry point to the electron transport chain. NADH, produced by citric acid cycle, or tricarboxylic acid cycle, is oxidized by complex I. The released electrons are transported through complex III and cytochrome c (Cyt c) to complex IV. Complexes I, III and IV all transport protons from matrix to intermembrane space, thus building the electrochemical gradient. Finally, the protons are able to flux back to matrix through ATP synthase and the energy released is stored by phosphorylation of ADP to ATP. Lower part: Complex II may act as the second entrypoint to the electron transport chain. Complex II accepts succinate, which is an intermediate product of tricarboxylic acid cycle, and oxidizes it to fumarate, reducing ubiquinone in the process.

4.11.1.2 Complex II

Complex II (CII) or succinate dehydrogenase (SDH) is the second entry point to the ETC (Figure 7). CII is unique among the respiratory complexes in two ways: first, it consists of four protein subunits that are all encoded by nuclear genes (Sun et al., 2005). Second, CII is the only subcomplex of the ETC which functions in both the TCA cycle and respiratory chain. In the TCA cycle, it oxidizes succinate to fumarate and reduces ubiquinone to ubiquinol at the same time, thus connecting the TCA cycle and respiratory chain.

4.11.1.3 Complex III

CIII or ubiquinol:cytochrome c oxidoreductase is a dimer that is constructed from 11 proteins, of which one is encoded by mitochondrial DNA (cytochrome b) and the rest by nuclear genes (Iwata et al., 1998). It oxidizes ubiquinol produced by CI and CII and reduces two molecules of cytochrome C. At the same time, it transfers four protons from the matrix to the intermembrane space, contributing to the electrochemical gradient (Zhang et al., 1998).

4.11.1.4 Complex IV

CIV or cytochrome C oxidase is the terminal oxidizer of the ETC. It accepts electrons from cytochrome C and uses them to reduce molecular oxygen to water. This is done with the help of two copper centers and heme sites in the complex (Tsukihara et al., 1995; Tsukihara et al., 1996). In addition, it transports four protons across the inner membrane, building up the electrochemical gradient even more. CIV is composed of 13 subunits, three of which are encoded by mitochondrial DNA and ten by nuclear genes.

4.11.1.5 ATP synthase

Complex V or ATP synthase forms an ion channel between the intermembrane space and the matrix (Abrahams et al., 1994). Through this channel protons are able to flow back to the matrix and the energy released in this flux is used to phosphorylate ADP with P_i, producing ATP. ATP synthase subunits in humans are encoded by two mitochondrial genes and 25 nuclear genes. In yeast, the F1Fo ATP synthase also functions in the forming of cristae folds (Davies et al., 2012).

Nutrient energy is not always used to phosphorylate ATP. Instead, it can be released as thermal energy if mitochondrial proton pumping from the ATP synthase is uncoupled. In brown adipose tissue, this uncoupling is done by thermogenin (Aquila et al., 1985). In total, five putative uncoupling proteins have been reported in mammals, but their function is not well established. Also, artificial uncoupling proteins, such as 2,4-dinitrophenol exists (Tainter et al., 1934). They have been used as weight-loss agents, with limited success due to their rather narrow therapeutic window.

4.11.2 ENERGY METABOLISM

Mitochondria release energy from pre-metabolized nutrients by the TCA cycle. Acetyl-CoA, which is produced by the metabolism of glucose, amino acids and fatty acids, is fed to the TCA cycle. The TCA cycle metabolizes Acetyl-CoA into NADH, which is fed into the OXPHOS system via CI. Also, oxidization of succinate, an intermediate product of the TCA cycle, to fumarate, is done by CII and feeds electrons to the respiratory chain. The whole TCA cycle occurs in the matrix. In addition to the TCA cycle, mitochondria take part in the urea cycle and beta-oxidation of fatty acids. Amino acid catabolism in most cases involves a deamination, in which an amino group is moved from the amino acid to alpha-ketoglutarate, which forms glutamate. Glutamate is again degraded into alpha-ketoglutarate, which enters the TCA cycle, and the amino group, which is processed in urea cycle. In the urea cycle, mitochondria convert amino groups from NH_4^+ into citrulline and inorganic phosphate, which are metabolized further in the cytosol into urea. In mammals, the urea cycle occurs mostly in liver and to a lesser extent in kidneys. As a by-product, two NADH molecules are formed and the total amount released is more than what is consumed. Not every amino acid is degraded by deamination: in some cases, uric acid or ammonia is formed. In beta-oxidation of fatty acids, fatty acids are first activated in the cytosol and then transported into mitochondria via the carnitine-acylcarnitine translocase, where chains of fatty acids are split into acetyl-CoA and can be fed into the TCA cycle.

4.11.3 OTHER FUNCTIONS OF MITOCHONDRIA

Mitochondria have various functions in addition to energy metabolism: they take part in steroid synthesis, iron-sulfur cluster formation and heme synthesis. Mitochondria also take part in apoptosis and iron and copper metabolism.

4.12 MITOCHONDRIAL DNA AND DISEASE

Mitochondrial diseases are a heterogeneous group of diseases, here restricted to include the malfunction of the respiratory chain. The causes are genetic, either in the genes encoded by nuclear 0r mitochondrial genomes. Mitochondrial myopathy, the most classic mitochondrial disease, is characterized by abnormalities in muscle biopsy samples: ragged red fibers, cytochrome C oxidase (COX) negative fibers on COX-SDH histochemical analysis and abnormal mitochondria on electron microscopy.

Another common feature of mitochondrial disease is abnormalities in mtDNA: deletions, point mutations and depletion. Also, duplication of part of mtDNA is a particular mutation type occasionally seen in patients, but usually together with deletions (Poulton et al., 1989a; Poulton et al., 1989b). mtDNA abnormalities can also be divided into acquired and inherited. Inherited mtDNA abnormalities, are caused by a direct alteration of mtDNA, such as the point mutation m.3243A→G, the most common cause of mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) and mitochondrial diabetes mellitus (MDD) (Goto et al., 1990; van den Ouweland et al., 1992). Acquired defects, such as multiple deletions and mtDNA depletion, are caused by malfunction in mtDNA replication, and nucleotide pool imbalances, for example. The mtDNA replication defects are due to nuclear gene mutations, such as in *POLG1* or *C10ORF2* (Spelbrink et al., 2001; Suomalainen et al., 1992a; Suomalainen et al., 1997; Van Goethem et al., 2001). However, toxic substances such as zidovudine, a nucleoside analog reverse-transcriptase inhibitor, can also affect mtDNA replication and cause mtDNA depletion (Arnaudo et al., 1991; Lewis et al., 1994). mtDNA depletion can be caused by *TYMP* mutations, which affect the nucleotide salvage pathway (Nishino et al., 1999). For example, a common cause of PEO is mutations in *POLG1*, leading to malfunction of POLG and multiple mtDNA deletions (Graziewicz et al., 2004; Van Goethem et al., 2001).

Grouping mitochondrial diseases based on the mtDNA rearrangements is relevant only in the historical context as the rearrangements were published before it was possible to study the mutations or genes behind the diseases (Bohlega et al., 1996; Holt et al., 1988; Moraes et al., 1989; Zeviani et al., 1989). Grouping mtDNA diseases based on mtDNA rearrangements is misleading: for instance, mitochondrial neurogastrointestinal encephalopathy (MNGIE), may exhibit either multiple mtDNA deletions or mtDNA depletion (Hirano et al., 1998). Classification of mtDNA diseases based on the mutated gene, is not helpful as mutations in the same gene, or even the same mutation, may exhibit great heterogeneity in the clinical presentation (Tang et al., 2011; Tzoulis et al., 2006). Due to the heterogeneous nature of mitochondrial diseases, no good classification of mitochondrial disease exists and most probably will not exist in near future.

4.13 DISEASES CAUSED BY *POLG1* MUTATIONS

POLG1 mutations are an important cause of neurodegenerative phenotypes, ranging from mitochondrial recessive ataxia syndrome (MIRAS) to parkinsonism (Luoma et al., 2004; Luoma et al., 2007; Van Goethem et al., 2004). Also, POLG1 with proofreading deficiency causes an artificial premature aging phenotype in mice (Trifunovic et al., 2004). The proofreading deficiency in POLG1 leads to neural and hematopoietic progenitor cell dysfunction early in embryogenesis, but manifests as a respiratory chain dysfunction in non-dividing tissues in old age (Ahlqvist et al., 2012). The clinical spectrum of *POLG1* mutations is heterogeneous. Even the same mutations in *POLG1* can cause different phenotypes, as is evident in the case of Alpers' disease and MIRAS. Also, the age of onset and clinical manifestations of disease show great variability. This clinical heterogeneity is a pronounced feature of POLG1 diseases and the heterogeneity is not as evident in other syndromes caused by malfunction of the mtDNA replication machine. For example, IOSCA, which is caused by a mutation in *Twinkle*, has a nearly similar clinical manifestation in every patient (Koskinen et al., 1994b). However, this heterogeneity of *POLG1* diseases might be a biased finding due to the higher frequency of *POLG1* mutations. *Twinkle* mutations have been reported in Alpers' disease and PEO with parkinsonism (Baloh et al., 2007; Sarzi et al., 2007; Vandenberghe et al., 2009).

4.13.1 MITOCHONDRIAL RECESSIVE ATAXIA SYNDROME

Mitochondrial recessive ataxia syndrome (MIRAS) was first identified in 2001 in a Finnish family and its genetic cause was found to be in *POLG1* (Rantamäki et al., 2001; Van Goethem et al., 2004). The common occurrence and ancient pan-European origin of the mutation was discovered by Hakonen in 2005 and the syndrome was named MIRAS (or MSCA-E, by Winterthun). Later, it has been described in patients from Norway, Belgium, Australia, New Zealand and United Kingdom (Hakonen et al., 2007a; Hakonen et al., 2005; Van Goethem et al., 2004; Winterthun et al., 2005). MIRAS is caused by *POLG1* mutations, most commonly the homozygous mutations c. 2243G→C *in cis* with c.3428A→G, leading to p.Trp748Ser + p.Glu1143Gly amino acid changes, the compound heterozygous mutations c. 2243G→C *in cis* with c.3428A→G and, on the other allele, c. 1399G→A, which respectively leads to p.Trp748Ser + p.Glu1143Gly and p.Ala467Thr amino acid changes (Figure 5). The carrier frequency of these disease alleles is high in Finland and Norway: 0.8% for c. 2243G→C *in cis* with c.3428A→G allele in Finland and 0.5% for combined c. 2243G→C *in cis* with c.3428A→G and c. 1399G→A alleles in Norway (Hakonen et al., 2005; Winterthun et al., 2005). In Italian, British and Spanish ataxia patient cohorts the high frequency of c. 2243G→C *in cis* with c.3428A→G and c. 1399G→A alleles

has not been replicated, but patients have been described in all Caucasian populations (Cagnoli et al., 2008; Craig et al., 2007; Hakonen et al., 2007a; Hakonen et al., 2005; Pelayo-Negro et al., 2012). All European patients share a common core haplotype in *POLG1*, indicating a common founder (Hakonen et al., 2007a; Hakonen et al., 2005).

The clinical features of MIRAS resemble other mitochondrial ataxias closely and differential diagnoses are not possible based solely on clinical signs (Table 1) (Hakonen et al., 2005; Rantamäki et al., 2001; Van Goethem et al., 2004; Winterthun et al., 2005). The typical age of onset for MIRAS varies from five years to 50 years. The course of MIRAS is variable: in Norway the median survival is 26 years after diagnosis for patients homozygous for c. 2243G→C in cis with c.3428A→G mutations in *POLG1* (Tzoulis et al., 2006). Patients, who are compound heterozygous for c. 2243G→C in cis with c.3428A→G mutations and c. 1399G→A have a worse prognosis as their median lifespan after diagnosis in Norway is just six years (Tzoulis et al., 2006). However, in Finland the life expectancy is not as limited (Suomalainen A, unpublished observation). Patients with age of onset under 20 years usually present with epileptic seizures or migraine-like headaches and distortion of vision, whereas adults present with gait disturbances and sensory polyneuropathy (Hakonen et al., 2005). Common symptoms in the early stages of disease are ataxia, neuropathy and lack or decrease in the tendon reflexes in lower limbs. Later, involuntary movements, dysarthria, dysphagia, nystagmus, psychiatric symptoms (depression, anxiety and hallucinations) and cognitive retardation may also be present. Epileptic seizures are prevalent, but not with every patient. In particular, adult-onset MIRAS may not cause epilepsy. Epileptic seizures are usually treatment resistant, may require general anesthesia and usually are the prelude to the patients' demise. Epilepsy presents a clinical challenge, as sodium valproate, a common anticonvulsant, is toxic to liver for patients with *POLG1* mutations and may lead to acute liver failure and ultimately liver transplantation (Ferrari et al., 2005; Tzoulis et al., 2006). In summary, a typical MIRAS patient has an onset in the third decade of life, with sensory polyneuropathy or gait disturbances and later progressive ataxia. Later, the patient may develop epilepsy. However, the clinical phenotype is highly variable. At the moment, no animal models of MIRAS have been published.

A surprising feature of MIRAS is that it does not necessarily exhibit the typical findings for mitochondrial disease: serum lactate is normal (except in some juvenile-onset cases) and muscle biopsy samples do not always show mtDNA deletions or depletion (Hakonen et al., 2005; Van Goethem et al., 2004). A minor amount of COX negative fibers can be, however, seen in the muscle biopsy samples. Patients might have an increase in serum pyruvate. Nerve biopsy samples from the sural nerve show a drastic decrease in large myelinated fibers. In electroneuromyography, MIRAS patients have a clear

sensory axonal polyneuropathy, with a slight motor component. In the early stages of MIRAS, patients may not have any changes in their brain magnetic resonance imaging (MRI). When the disease progresses, the patients' brains start to show bilateral cerebellar white matter changes in brain MRI, changes in basal ganglia, white matter changes and generalized brain atrophy.

4.13.2 ALPERS' SYNDROME

Alpers' syndrome is the most severe manifestation of POLG1 defects (Ferrari et al., 2005; Naviaux and Nguyen, 2004; Naviaux and Nguyen, 2005; Nguyen et al., 2005). Alpers' syndrome is a recessive disease, caused by several different homozygous or compound heterozygous *POLG1* mutations, c. 1399G→A, leading to p.Ala467Thr amino acid change, being the most common and c. 2243G→C *in cis* with c.3428A→G, leading to p.Trp748Ser + p.Glu1143Gly amino acids changes (in other words, the same mutation as found in MIRAS, thus underlining the heterogeneity of clinical manifestations in POLG1 diseases) being the second most common (Figure 5) (Tang et al., 2011). Some MIRAS patients are compound heterozygous for c. 2243G→C *in cis* with c.3428A→G and c. 1399G→A mutations.

Alpers' syndrome is an autosomal recessive disease, with onset in early infancy or childhood, usually before five years of age (Ferrari et al., 2005; Isohanni et al., 2011; Naviaux and Nguyen, 2004). Alpers' syndrome is characterized by four symptoms: psychomotor retardation, refractory epileptic seizures, cortical blindness and liver disease with micronodular cirrhosis (Table 1). Liver involvement is not always present (Huttenlocher et al., 1976). The infants are born healthy and the very first developmental milestones are normal. Often, the disease onset is after febrile illness, such as otitis media or diarrhea. In most cases, however, the presenting symptom is epileptic seizure. Other clinical symptoms that may develop are encephalopathy, ataxia, hypotonia and occasional elevation in serum lactate. From the onset of the disease, the symptoms and epileptic seizures become more severe, acute liver failure develops, which leads to the patients death within a couple of years.

mtDNA depletion and respiratory chain complex deficiency may be present, but not necessarily (de Vries et al., 2007; Isohanni et al., 2011). Also, muscle biopsy samples can lack any findings suggesting mitochondrial disease (Isohanni et al., 2011). The patients may have changes in brain MRI, but usually at the time of onset the MRI does not show any abnormalities. In the later course of the disease, cortical and hippocampal atrophy may be present, as well as focal lesions (Isohanni et al., 2011).

In post-mortem pathological examination, the patients have cerebellar atrophy, with patchy dropout of Purkinje cells (Ferrari et al., 2005; Naviaux and Nguyen, 2004). They also have atrophy of the cerebral cortex, visual cortex and occipital cortex. Also, the patients have neuronal loss in the basal ganglia, thalamus and hippocampi (Isohanni et al., 2011). In the liver, micronodular cirrhosis, hepatocyte dropout, microvesicular fat and bile ductural proliferation is seen.

The biochemical effect of several different amino acid changes, connected to Alpers' syndrome, has been studied (Chan et al., 2005; Luoma et al., 2005). Luoma and co-workers described the effect of p.Ala467Thr, p. Arg627Gln, p. Arg627Trp, p. Gln1236His and p. Arg627Gln + p. Gln1236His amino acid changes. Later, Chan and co-workers studied the effect of POLG1 p.Ala467Thr. The amino acid changes Luoma and co-workers studied were chosen because they were found in a family with ataxia-myopathy syndrome. The most severely affected member of the family had mutations leading to p.Ala467Thr, p. Arg627Gln and p. Gln1236His amino acid changes. Both studies found that the intrinsic polymerase activity and processivity of the p.Ala467Thr POLG1 were diminished (Chan et al., 2005; Luoma et al., 2005). Also, the interaction with the accessory subunit POLG2 and the ability of POLG2 to stimulate the function of the catalytic core was depleted. Although the results were similar, there were clear differences in the severity of the phenotype: Chan et al reported an almost complete lack of activity, whereas Luoma and co-workers described an enzyme that is clearly affected but partially functional. Luoma and co-workers described also p. Arg627Gln and p. Arg627Trp POLG1 enzymes, which did not exhibit a clear biochemical phenotype, whereas p. Gln1236His had higher enzymatic activity than the wild type enzyme (Luoma et al., 2005). No animal models of Alpers' syndrome have been published.

Table 1. *Comparison of mitochondrial ataxias. + present, - absent, ? unknown, RRF Ragged red fibers, COX- Cytochrome c oxidase deficient fibers*

	MIRAS	Alpers'
Age of Onset	5-50 yrs	0-4 yrs
Presenting symptom	Gait disturbances / Epilepsy	Intractable epilepsy, hypotonia
Babinski sign	-/+	?
Deep tendon reflexes	-	-
Epilepsy	+/-	+
Nystagmus	+/-	?
Dysarthria	+	-
Dysphagia	+/-	?
Hearing loss	-	-
Psychiatric symptoms	Depression, anxiety, paranoia	-
Cognition	Cognitive defects	?
Sensation	Decreased for joint position, vibration	?
Neuropathy	Mostly sensory, small motor component occasionally	?
Ophthalmoplegia	+/-	-
Muscle mtDNA	Few or no COX-negative fibers. Small amount of multiple mtDNA deletions, occasional mild depletion	mtDNA depletion
References	(Rantamäki et al., 2001; Van Goethem et al., 2004)	(Huttenlocher et al., 1976; Naviaux and Nguyen, 2004)

IOSCA	SANDO	ARSACS	Friedreich's
0-3 yrs	~20 yrs	1 yrs	10-51 yrs
Hypotonia, athetosis	Gait disturbances	Ataxia, Pyramidal tract symptoms	Ataxia
+	-	+	+
-	-	Ankle –	-
+	-	-	-
-	-	+	+
-	-	+	+
-	?	?	?
+	+	?	-
-	Depression, bipolar	-	-
Psychomotor retardation	Cognitive defects	Lower IQ	?
Proprioception, tactile decreased	Joint, vibration decreased	Joint, vibration decreased	Joint, vibration decreased
Sensory axonal		Sensory axonal	Sensory axonal
-	+	-	-
Normal	RRF, COX-, mtDNA deletions	Normal	Normal
(Koskinen et al., 1994a; Koskinen et al., 1994b)	(Fadic et al., 1997; Mancuso et al., 2004; Van Goethem et al., 2003)	(Bouchard et al., 1978)	(De Michele et al., 1994; Harding, 1981)

4.13.3 SENSORY ATAXIC NEUROPATHY, DYSARTHRIA AND OPHTHALMOPARESIS

Sensory ataxic neuropathy, dysarthria and ophthalmoparesis (SANDO) is a mitochondrial ataxia, caused by several different mutations in POLG1 (Fadic et al., 1997). c. 1399G→A and c. 2243G→C mutations in POLG1 are the most

common mutations (Schulte et al., 2009). Clinically, SANDO is closely related to late-onset MIRAS: the disease onset is around 20 years of age orlater and the presenting symptom is weakness in lower limbs or gait disturbances (Table 1) (Fadic et al., 1997; Mancuso et al., 2004; Van Goethem et al., 2003). Additionally, ataxia, dysarthria, neuropathy, external ophthalmoplegia, ptosis, lack of deep tendon reflexes, diminished vibration and position sense may also be present. In comparison to MIRAS, epilepsy has not been reported in SANDO and muscle atrophy is a more prominent feature. The disease progression is steady and leads to death of patients in their sixth decade of life. The most common cause of death is respiratory failure, again a clearly different feature from MIRAS. Laboratory examinations do not provide any insight into the diagnosis: serum lactate and creatine kinase are normal or occasionally mildly elevated. Muscle biopsy samples have varying findings: some patients have ragged red fibers (RRF, muscle fibers which have accumulated abnormal mitochondria), and multiple mtDNA deletions, whereas some do not. In brain MRI, signs of thalamic lesions and brain stem atrophy may be present. No animal models of SANDO have been published.

4.13.4 PEO WITH PARKINSONISM

POLG1 mutations cause multiple different syndromes, one of which is autosomal dominant PEO with parkinsonism (POLG1-PD) (Luoma et al., 2004). POLG1-PD has typical features for PEO: onset in 20-30's with external ophthalmoplegia, ptosis and possibly sensory polyneuropathy (Chalmers et al., 1996; Checcarelli et al., 1994; Luoma et al., 2004; Moslemi et al., 1999; Siciliano et al., 2001). Parkinsonian features develop soon after or later, usually before the sixth decade. Tremor, rigidity and hypokinesia can be unilateral or bilateral, again different from idiopathic PD. Parkinsonian features develop slowly and show a good response to levodopa treatment. Usually, no Lewy bodies or α-synuclein pathology are found, although some cases have been described in which Lewy bodies are found in substantia nigra (SN) and adjacent structures (Betts-Henderson et al., 2009; Moslemi et al., 1999). Otherwise, the neuropathological changes are similar to other POLG1 syndromes: sensory areas of spinal cord, cerebellum and brain stem show extensive neuronal death. It should also be noted that two case reports have been published, in which *C10ORF2* mutation c. 1121G→A caused PEO with parkinsonism (Baloh et al., 2007; Vandenberghe et al., 2009).

4.14 OTHER CAUSES OF MITOCHONDRIAL ATAXIA

4.14.1 INFANTILE-ONSET SPINOCEREBELLAR ATAXIA

Infantile-onset spinocerebellar ataxia, IOSCA, is a progressive ataxia syndrome originally known as OHAHA (ophthalmoplegia, hypotonia, ataxia, hypacusis and athetosis) (Koskinen et al., 1994b). IOSCA is part of the Finnish disease heritage, with no patients elsewhere. IOSCA is caused by a mutation in mitochondrial DNA helicase, TWINKLE (encoded by *C10ORF2*). Two mutations are found: recessive c. 1523 A→G and compound heterozygous c. 1523 A→G with c.952 G→A, leading to p. Tyr508Cys and p. Tyr508Cys + p. Ala318Thr amino acid changes, respectively (Hakonen et al., 2007b; Nikali et al., 2005).

The onset of IOSCA is early in life, after an uneventful birth and normal first milestones, at latest at the age of one and half years. (Table 1) (Koskinen et al., 1994a; Koskinen et al., 1994b). Again, as in Alpers' syndrome, the first symptoms develop slowly or more quickly during febrile disease. The presenting symptoms are muscular hypotonia, ataxia and athetosis, or slowly progressing gait disturbances. The symptoms develop into ophthalmoplegia, optic atrophy and rapidly progressing deafness, which reaches almost complete deafness before the age of seven years. Patients have neuropathy and their muscles in lower limbs become atrophied. Some patients learn to walk with support, whereas some do not. The cognitive capacity of the patients is normal, but during the progression of disease becomes more limited. Almost all patients develop epilepsy, which is usually the cause leading to their demise. The epilepsy, like in other mitochondrial diseases, is severe and hard to treat. Life expectancy may be limited, although some patients are still alive in their 50's.

Laboratory examinations do not provide any relevant information for diagnosis. In electroneuromyography patients have sensory axonal neuropathy. Brain MRI is normal in the first stages of the disease but during epileptic seizures, stroke-like episodic changes can be seen (Koskinen et al., 1995; Lonnqvist et al., 2009). Patients' muscle biopsy samples are also normal and they have no mtDNA deletions or disruptions in respiratory chain components (Nikali et al., 2005). In liver and brain tissue samples (cerebral cortex and cerebellum) however, patients have tissue specific mtDNA depletion and CI and CIV deficiency (Hakonen et al., 2008b).

In neuropathological examination, the patients have moderate atrophy of cerebellum and brain stem (Hakonen et al., 2007b; Lönnqvist et al., 1998). In spinal cord, they have severe atrophy of posterior aspects of the spinal cord, especially posterior spinocerebellar columns and spinal ganglions. In the

cerebellum, loss of Purkinje cells and severe destruction of the nucleus dentatus are the most prominent findings. In the mesencephalon, loss of neurons in SN is found.

The biochemical effect of p. Tyr508Cys amino acid change has been studied with *in vitro* assays (Hakonen et al., 2008b). The p. Tyr508Cys TWINKLE had higher helicase unwinding activity than the wild-type enzyme, but overexpression of p. Tyr508Cys TWINKLE did show a minor increase in mtDNA copy number. However, no catalytic defect was detected. Currently no animal models of IOSCA exist.

4.14.2 FRIEDREICH'S ATAXIA

Friedreich's ataxia is an autosomal recessive ataxia syndrome, which is caused by mutations in the *FXN* gene (Campuzano et al., 1996). The most common mutation is a homozygous expansion of a GAA-repeat in the first intron of *FXN*, which accounts for 95% of cases. In a small minority of cases, the GAA-repeat is compound heterozygous with a point mutation in *FXN* (Cossee et al., 1999). The age of onset is in most cases in puberty, although the disease may develop as early as two years of age or later, at the age of 50 (Table 1) (De Michele et al., 1994; Harding, 1981). The presenting symptoms are gait disturbances and ataxia, which develop into tremor and loss of balance. The patients lose their ability to walk without support usually within 10-15 years. Also, dysarthria and dysphagia develop. Patients have hypertrophic cardiomyopathy and repolarisation disturbances in heart, although this does not cause clinical symptoms in most cases (Casazza and Morpurgo, 1996). Neither mtDNA abnormalities nor COX deficient muscle fibers have been found. Neuropathological findings resemble other mitochondrial ataxias: axonal sensory polyneuropathy, atrophic posterior columns in spinal cord, as well as spinocerebellar tracts [Reviewed in (Koeppen, 2011)]. Nucleus dentatus is severely affected in cerebellum, but cerebellar cortex or Purkinje cells are mildly affected. Motor tracts are not affected in early phases of the disease, but later show some atrophy, probably due to the dying-back process. Several different mouse models of Friedreich's ataxia have been produced: complete knock-out of *FXN* is embryonic lethal, whereas knock-in models with variable GAA-repeat expansions lead to decrease in *FXN* expression (Cossee et al., 2000; Miranda et al., 2002). Time-course analysis of pathological changes in a specific neuronal knock-out of *FXN* has shown that the damage in distal root ganglia is the primary insult, which leads to degeneration in Clarke's column and other areas of spinal cord and brain (Simon et al., 2004). Friedreich's ataxia is an extremely rare disease in Finland, with just six reported patients (Juvonen et al., 2002). However, Friedreich's ataxia is more common elsewhere.

4.14.3 AUTOSOMAL RECESSIVE SPASTIC ATAXIA OF CHARLEVOIX-SAGUENAY

Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) was first described in French-Canadian regions of Quebec (Bouchard et al., 1978). ARSACS is a slowly progressing ataxia syndrome, with dysarthria, spasticity, wasting of distal muscles, nystagmus and mitral valve prolapse (Table 1). ARSACS is not only found in Quebec, but also in Italy and Japan (Criscuolo et al., 2004; Grieco et al., 2004; Ogawa et al., 2004). The disease onset is in early childhood, but late-onset disease has also been described. The patients rarely walk at all. Despite these features, the disease progression is slow but still leads to reduced life expectancy. ARSACS is caused by mutations in the *SACS* gene and although a variety of mutations in this gene have been described, no definite correlations between genotype and phenotype exist (Engert et al., 2000). SACSIN, encoded by *SACS*, is a protein that affects mitochondrial dynamics: knock-out and knock-down of *SACS* in mice leads to a more interconnected mitochondrial network, diminished membrane potential and ultimately lack of dendrites (Girard et al., 2012). Also, *SACS* knock-out mice are born normal but later develop a progressive loss of Purkinje cells in the cerebellum. Neuropathological findings in humans are similar: a progressive loss of Purkinje cells and cerebellar atrophy are the most prominent findings [Reviewed in (Bouchard et al., 1998)]. Although SACSIN is a mitochondrial protein, no disruption in respiratory chain or mtDNA has been described.

4.15 PROGRESSIVE EXTERNAL OPHTHALMOPLEGIA

Progressive external ophthalmoplegia (PEO) is a classical mitochondrial syndrome, which is characterized by diminished eye movements and ptosis. PEO is a feature in multiple mitochondrial disorders, with many different causes (Table 2). Patients develop PEO usually between 20-40 years of age. Together with ptosis and sensory polyneuropathy, diminished eye movements are usually the presenting symptom. The variety of other clinical symptoms connected to PEO is diverse: ataxia, myopathy, psychiatric disorders, premature menopause, primary ovarian failure, hypogonadism, parkinsonism and a plethora of other symptoms have been reported (Bohlega et al., 1996; Lundberg, 1962; Melberg et al., 1996; Moraes et al., 1989; Suomalainen et al., 1992a; Suomalainen et al., 1997; Suomalainen et al., 1992b; Van Goethem et al., 1997). When mitochondrial disease is suspected, histological analysis of a muscle biopsy sample is considered to be the gold standard for diagnosis, before molecular diagnosis. In PEO, muscle biopsy samples have typical changes for mitochondrial disease: RRFs, COX negative

fibers and decreased enzyme activity for respiratory chain enzymes. Mitochondrial DNA deletions, depletion or point mutations may be found in muscle samples. The deletions can be either multiple deletions in muscle or single large deletions in all tissues (Bohlega et al., 1996; Holt et al., 1988; Moraes et al., 1989; Zeviani et al., 1989). PEO with single deletions is sporadic whereas PEO with multiple mtDNA deletions is caused by autosomal genes, in a recessive or dominant manner (arPEO, adPEO).

The last decade has opened the molecular basis of PEO and revealed several different modes of transmission and disease loci: mendelian or maternal inheritance, autosomal recessive and dominant PEO, point mutations and single deletions in mtDNA, and mutations in nuclear genes have all been reported (Hudson et al., 2008; Kaukonen et al., 2000; Longley et al., 2006; Moraes et al., 1989; Spelbrink et al., 2001; Takata et al., 2011; Tyynismaa et al., 2012; Tyynismaa et al., 2009; Van Goethem et al., 2001; Verma et al., 1996). Mutations in *ANT1* (HUGO name *SLC25A4*), *Twinkle (C10Orf2)*, *POLG1, POLG2*, *TK2, OPA1* and *RRM2B* are all connected to PEO. However, mutations in *POLG1* and *TK2* are the only known cause of arPEO.

Table 2. *Comparison of PEO syndromes. Mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS), autosomal dominant / recessive progressive external ophthalmoplegia (adPEO/arPEO), mitochondrial neurogastrointestinal encephalomyopathy (MNGIE).*

	MELAS	**Kearns-Sayre Syndrome, sporadic PEO**	**adPEO/arPEO**	**MNGIE**
Genetic cause	mtDNA m.3243A→G, *MTTL1* gene	mtDNA deletion	*POLG1*,*POLG2*, *Twinkle*, *RRM2B*, *OPA1*,*TK2*, *ANT1*	*TYMP* and *RRM2B* mutations
mtDNA rearrangements	-	Single large mtDNA deletion	Multiple mtDNA deletions	mtDNA depletion and deletions
Age of Onset	Any age	Early teenage	Early adulthood	Juvenile
Typical symptoms	Epileptic seizures, stroke-like episodes, headache, PEO, diabetes, deafness	Retinitis pigmentosa, dilated cardiomyopathy, PEO	Ptosis, myopathy, PEO, exercise intolerance	Gastrointestinal dysfunction, muscle atrophy, PEO, nausea
Other symptoms	Exercise intolerance, encephalopathy, lactic acidosis	Cardiac conduction blocks, ataxia, hearing loss, dementia	Polyneuropathy, parkinsonism, depression	Polyneuropathy, leukoencephalopathy, cachexia
References	(Goto et al., 1990; Pavlakis et al., 1984)	(Kearns, 1965; Lestienne and Ponsot, 1988)	(Moraes et al., 1989; Suomalainen et al., 1992a; Suomalainen et al., 1997; Van Goethem et al., 2001; Zeviani et al., 1989)	(Bardosi et al., 1987; Hirano et al., 1994; Taanman et al., 2009)

4.16 MITOCHONDRIA AND PARKINSONISM

Parkinson's disease (PD) is the second most common neurodegenerative disease in the world, with a prevalence of 166/100000 in Finland. PD is

characterized by three clinical symptoms: rigidity, tremor and hypokinesia. These symptoms are the main motor symptoms associated with PD. These motor symptoms develop quite late in the course of disease, delaying the diagnosis. Non-motor symptoms, such as disturbances of sleep, incontinence, constipation and impaired ability to smell, develop earlier but as they are rather unspecific they do not help in the diagnosis. The median age of onset for PD is usually in the sixth decade of life (Table 3). No diagnostic laboratory tests or imaging examinations exist and the diagnosis is based on the clinical signs. Single-photon emission computed tomography and positron emission tomography may aid in the diagnosis, but otherwise laboratory examinations and imaging studies are done to rule our other possible causes for the symptoms. The gold standard for PD diagnosis is found post mortem: lack of neurons in the SN and more specifically, Lewy bodies in the remaining neurons of the SN. Lewy bodies are protein aggregates, which are composed mainly of misfolded alpha-synuclein. When motor symptoms develop, about 60% of SN dopaminergic neurons have died. This causes a problem for both diagnosis and treatment of the disease: the diagnosis is made late in the course of the disease and the treatment is only to relieve symptoms, not to reverse the course of the disease.

During the last decades there has been an enormous effort to understand the pathogenesis of PD. Since the first description of an Italian family with hereditary PD, caused by mutations in *SNCA*, several loci have been described (Lill et al., 2012; Polymeropoulos et al., 1997; Saad et al., 2011; Simon-Sanchez et al., 2009). Currently 20 different loci, which cause either recessive or dominant PD or alter the risk of sporadic PD, have been confirmed. These loci are connected to a heterogeneous set of pathways. However, some noteworthy pathways have emerged: The α-synuclein pathway, the leucine-rich repeat kinase 2 (LRRK2) pathway and the mitochondrial pathway, consisting of PD caused by mutations in *PINK1*, *Parkin*, *DJ-1* and *POLG1*.

Mitochondrial dysfunction as a cause of PD was proposed long before the modern age of genetics. PD patients have CI deficiency in multiple tissues: platelets, muscle, lymphocytes and SN (Bindoff et al., 1989; Haas et al., 1995; Krige et al., 1992; Parker et al., 1989; Schapira et al., 1989; Yoshino et al., 1992). Mitochondrial toxins and CI inhibitors rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), can cause PD-like disease (Betarbet et al., 2000; Langston et al., 1983; Langston and Ballard, 1983). These substrates are also widely used to produce animal models for PD. It was therefore not a surprise when PARK2, PARK6 and PARK7 loci, causing autosomal recessive juvenile Parkinson's disease (ARJPD) were linked to mitochondrial function.

ARJPD is a subgroup of hereditary PD, which is characterized by early onset between 20-40 years of age, bilateral symptoms, slow disease progression and critically the lack of Lewy bodies (Table 3) (Albanese et al., 2005; Dekker et al., 2003; Ibáñez et al., 2006; Lücking et al., 2000; Mori et al., 1998; van de Warrenburg et al., 2001). ARJPD is caused by mutations in *PARK2*, encoding parkin. (Kitada et al., 1998) Shortly after *PARK2* mutations were described, mutations in *DJ1* and *PINK1* were reported to cause similar early-onset, recessive PD (Bonifati et al., 2003; Valente et al., 2004). In cultured cells and *D. melanogaster*, Parkin and PINK1 have been found to function in the same mitophagy pathway, as described in Figure 2. Parkin overexpression is able to rescue the PINK1 mitochondrial phenotype, but not reverse, supporting the function in the same pathway even further (Park et al., 2006). The function of DJ-1 is not well understood, but it relocates from cytosol to mitochondria under oxidative conditions and the loss of DJ-1 leads to mitochondrial fragmentation and impaired mitophagy (Canet-Aviles et al., 2004; Irrcher et al., 2010; Junn et al., 2009; Krebiehl et al., 2010; Thomas et al., 2011). Overexpression of PINK1 and PARKIN rescues the structural abnormality caused by DJ-1 deficiency, suggesting that they all operate in the same pathway (Irrcher et al., 2010).

Table 3. *Comparison of PD symptoms. PD Parkinson's disease, ARJPD Autosomal recessive juvenile Parkinson's disease, POLG1-PD PEO with parkinsonism,SN substantia nigra, ANS: autonomic nervous system*

	Idiopathic PD	**ARJPD**	**POLG1-PD**
Age of Onset	Median 60 yrs	20-40 yrs	30-60 yrs
Lewy bodies in SN	Yes, prevalent	No	Occasionally in SN
Bilateral / Unilateral onset of symptoms	Unilateral	Bilateral	Both
ANS symptoms	Yes	No	Not characterized
Levodopa response	Initially good	Well preserved	Well preserved
Parkinsonism progression	Quick	Slowly progressive	Slowly progressive
Cognitive defects	Commonly dementia	No	Occasionally
References	Reviewed in (Lees et al., 2009)	(Albanese et al., 2005; Dekker et al., 2003; Ibáñez et al., 2006; Lücking et al., 2000; Mori et al., 1998; van de Warrenburg et al., 2001)	(Betts-Henderson et al., 2009; Luoma et al., 2004)

The function of α-synuclein has been and is still largely unknown. However, strong evidence suggests that α-synuclein has a mitochondrial-connected function [For a review (Protter et al., 2012)]. It has a cryptic N-terminal mitochondrial targeting signal and is able to relocate from the cytosol to the mitochondrial inner membrane (Devi et al., 2008; Parihar et al., 2008). The exact signal that triggers the relocalization is not known. Alpha-synuclein alters the mitochondrial fusion / fission cycle and thus the mitochondrial morphology in dopaminergic human SH-SY5Y neuroblastoma cells and HeLa cells: overexpression of α-synuclein leads to decreased fusion

events and fragmentation whereas knockdown of α-synuclein causes mitochondrial elongation (Kamp et al., 2010; Nakamura et al., 2011). The fission inducing function of α-synuclein is independent from the native fission machinery (Nakamura et al., 2011). In addition, the PD associated, p. Ala53Thr α-synuclein induces mitophagy (Choubey et al., 2011). Alpha-synuclein knock-out mice also exhibit resistance to the CI inhibitor MPTP (Dauer et al., 2002).

LRRK2 mutations are a common cause of both autosomal-dominant and sporadic PD (Kachergus et al., 2005; Lill et al., 2012). However, the penetrance of the *LRRK2* mutations and clinical symptoms show more variability than a-synuclein (Kachergus et al., 2005; Papapetropoulos et al., 2006). *LRRK2* mutations have also been connected to other neurodegenerative phenotypes, such as amyotrophy, Meige dystonia syndrome and supranuclear gaze palsy (Chen-Plotkin et al., 2008; Ross et al., 2006a; Ross et al., 2006b). Like α-synuclein, the function of LRRK2 has remained elusive. A function in lysosomal trafficking has been suggested. LRRK2 is present mainly in the cytosol, but also in the outer membrane of mitochondria (Biskup et al., 2006). In *C. elegans* and *D. melanogaster*, LRRK2 has been shown to alter the susceptibility to rotenone induced CI deficiency, thus strengthening the mitochondrial link (Ng et al., 2009; Saha et al., 2009). Niu and Wang have recently demonstrated an even more direct connection of LRRK2 and mitochondrial function, using human cerebral cortex neurons, dopaminergic SH-SY5Y cells and rat primary neurons (Niu et al., 2012; Wang et al., 2012). They show that LRRK2 disrupts mitochondrial dynamics via the dynamin like GTPase DLP1, causing mitochondrial fragmentation. The effect required for the kinase-function of LRRK2 and the commonly altered p. Gly2019Ser and p. Arg1441Cys residues had even stronger phenotypes. The current data from PINK1, DJ-1, PARKIN, α -synuclein and LRRK2 related PD show a possible role for mitochondrial dynamics in PD pathogenesis, not just limited to ARJPD.

5 AIMS OF THE STUDY

The work for this study began in 2003, with a specific focus on the diseases caused by POLG malfunction. Two particular findings were the basis for this thesis: the recent characterization of MIRAS by our research group and collaborators and the role of *POLG1* mutations in both idiopathic and PEO-linked parkinsonism.

The questions we wanted to answer in this study were:

- Why only some patients with dysfunctional mtDNA replisome develop parkinsonism and others do not? Could this be explained by mesencephalonic changes or dysfunctional respiratory chain?
- Is it possible that the high carrier frequency of the MIRAS disease allele in Finland and other countries could cause MIRAS to manifest with a pseudo-dominant inheritance?
- Can we explain the clinical heterogeneity of MIRAS with the biochemical properties of p. Thr748Ser POLG1?
- Can we utilize existing biochemical data of POLG1 amino acid changes and the crystal structure of POLG1 to assess the structure-functional relationship for recessive POLG1 disease mutations?

6 MATERIALS AND METHODS

The methods used in this study are summarized briefly here and described in more detail in the original publications (I-IV).

This study was approved by the ethical review board of Helsinki University Central Hospital (Dnro: 43/13/3/04/2008 and 432/E9/2007) and the subjects or their relatives gave their written informed consent.

6.1 PATIENTS AND CONTROLS (I, II)

We collected autopsy samples from patients with mtDNA maintenance disorders. MIRAS (n=3) and POLG1-PEO-parkinsonism (n=2) with POLG1 mutations were compared to IOSCA (n=1) and adPEO (n=1), the latter not associated with parkinsonism. As controls we had samples from subjects, who died of non-neurological causes (n=3).

6.1.1 MIRAS PATIENTS

The presenting symptom for all MIRAS patients (n=3) was gait disturbance, but the age of onset varied: from childhood to early thirties. All patients developed ataxia, sensory polyneuropathy and epilepsy. Depression and anxiety were also present in all patients. All MIRAS patients were homozygous for mutations in *POLG1,* c. 2243G→C *in cis* with c.3428A→G, leading to p.Trp748Ser + p.Glu1143Gly amino acid changes.

6.1.2 POLG1-PD PATIENTS

Both patients developed typical signs of PEO in their third decade of life: ptosis, external ophthalmoplegia and sensory polyneuropathy. Later, they developed difficulties in swallowing. They also had a decrease in the strength of his neck muscles and one patient required a mechanical support. The ages for diagnosis of PD were 46 and 57 years of age. The presenting symptom of parkinsonism was rigidity and lack of associated movements in one patient and wide, unsteady gait and hypokinesia in another. Both were responsive to levodopa and although the parkinsonism progressed steadily in both patients, the course of the disease was mild. *POLG1* was sequenced in both patients: P4 was homozygous for c. 1402A→G and c. 3316G→A, leading to p. Asn468Asp and p. Ala1105Thr amino acid changes. P5 was heterozygous for

c.2864A→G 13906C→T mutation in *POLG1*, resulting in p.Tyr955Cys amino acid change.

6.2 MORPHOLOGICAL AND NEUROPATHOLOGICAL ANALYSIS (I, II)

Neuropathological examination was performed according to standard clinical practice and autopsies were performed 4-72h post mortem.

Neuropathological samples were studied after fixation in formalin embedding in paraffin. We used samples from brain, spinal cord and muscle from both patients and controls. We used 4-8 µm sections. We used hematoxylin-eosin and luxol fast blue-cresyl violet. In immunohistochemistry, we used various antibodies for: p62 (Clone D-3, Santa Cruz, CA, US), alpha synuclein (clone 42, BD Biosciences, US), TAU (Clone AT8, Innogenetics, Belgium), GFAP (MO761, 6F2; Dako Carpinteria, CA, US), SMI-311 (Sterberger Monoclonals Inc, Baltimore, MD, US) and AP-2 (M-4403, Sigma, St. Louis, MO, US).

In respiratory chain component immunohistochemistry we used antibodies against ND1 and AFG3L2 (Gift from Dr. Anne Lombes and Dr. Thomas Langer, respectively), NDUFS3 (MS110, Mitosciences, Eugene Oregon, US), NDUFA9 (MS 111, Mitosciences) of CI, 70 kDa Fp subunit (MS204, Mitosciences) against CII, UQCRC2 (MS304, Mitosciences) against CIII and COXII (MS405, Mitosciences) against CIV.

DAB was used as a visualization agent. The exact reaction conditions and antibody concentrations are described in articles I and II.

6.3 MTDNA ANALYSIS (I)

6.3.1 MTDNA POINT MUTATION LOAD

We extracted total DNA from muscle using standard laboratory methods.

We used PCR to amplify parts of the mtDNA control region (D-loop) and cytochrome *b* gene as described in study I. The amplified products were cloned into the Zero Blunt TOPO PCR cloning vector (Invitrogen) and we extracted plasmid DNA from the samples. For cytochrome *b* gene 25 000 nt and for D-loop 20 000 nt were sequenced by Sanger sequencing, per patient. We used Sequencher (Gene Codes Corporation, Ann Arbor, MI, US) software

to analyse the sequences. Each mutation was counted once, to evaluate *de novo* mutagenesis and to rule out clonal expansion of the same mutation.

6.3.2 MTDNA DELETIONS

We used long-range PCR to amplify an 8.3 kb part of mtDNA, located between nucleotides 8232-16496. Muscle mtDNA (from total DNA) was used as a template and we used Expand Long Template PCR System (Roche) with buffer system I. The annealing temperature was set to 63°C and we used two different extension times, 8 and 3 minutes, to favour the amplification of deleted mtDNA molecules. As a control we used samples from a patient with multiple mtDNA deletions and a normal control.

6.3.3 MTDNA LEVEL

We amplified a part of the cytochrome b gene (mitochondrial gene target) and compared its amplification product with the nuclear gene encoding amyloid precursor protein (APP) using TaqMan real-time quantitative PCR system, to quantify the amount of mitochondrial DNA. Total muscle DNA was used as a template. The reactions were done in triplicates. We used ABI PRISM 7000 Sequence Detection System. We created a standard curve using known copy numbers of standard plasmids containing the CytB or APP gene targets, and subsequently, relative quantity of mtDNA to APP.

6.4 *POLG1* SEQUENCING (I)

POLG1 mutations were screened from the patients by traditional Sanger sequencing. We analysed all exons of *POLG1*, as previously published (Van Goethem et al., 2001).

6.5 PROTEIN PREPARATION (III)

6.5.1 CONSTRUCTION OF RECOMBINANT BACULOVIRUSES

We constructed baculovirus transfer vectors carrying POLG1 cDNAs with c.2243G→C nucleotide change using QuikChange mutagenesis with Pfu DNA polymerase (Stratagene, Santa Clara, CA, US). Transfer vectors were purified and baculoviruses prepared as previously (Wang and Kaguni, 1999).

6.5.2 PURIFICATION OF RECOMBINANT POLG1

The method used has been described in detail (Luoma et al., 2005). In short, we infected *Sf9* (*Spodoptera frugiperda*) insect cells with baculoviruses carrying mutant POLG1. Cells were collected, homogenized and centrifuged to extract the cytoplasmic soluble fraction (Fraction I). This fraction was loaded on a phosphocellulose column, which was washed with potassium phosphate buffer. The resulting fractions were analyzed for activity using standard polymerase assay with gapped dsDNA. Active fractions were pooled (Fraction II) and incubated with ammonium sulfate overnight, dialysed to an ionic strength of 300-400 mM KCl (Fraction IIb) and loaded to nickel-nitrilotriacetic acid (Ni-NTA) resin. Again, active fractions were pooled (Fraction III) and loaded to glycerol gradients and centrifuged. Finally, the active fractions were pooled and stabilized by addition of glycerol to 45% (Fraction IV). Equivalent units of fraction IV enzyme were electrophoresed and stained using silver. The specific activity of the enzyme was measured using standard polymerase assay with gapped dsDNA.

6.6 BIOCHEMICAL ANALYSIS OF POLG FUNCTION (III)

6.6.1 MEASUREMENT OF DNA POLYMERASE ACTIVITY

DNA polymerase activity was measured on (1) DNase I-activated calf thymus DNA, (2) singly-primed M13 DNA and (3) poly r(A)· oligo d(T). For steady state kinetic values, we used 5-400 nM of DNase I-activated calf thymus DNA or 0.125-8 μM singly-primed M13 DNA. All assays were done at least twice with duplicate samples. For stimulation assays, four-fold excess of recombinant POLG2 was added to reaction mixtures. With one unit of standard activity, we refer to the amount of protein that catalyses the incorporation of 1 nm of deoxynucleoside triphosphate into acid insoluble material in 60 min at 30 ˚C using DNase I-activated calf thymus DNA as a substrate.

6.6.2 DNA BINDING ASSAYS

We used gel electrophoretic mobility-shift assay to measure DNA binding affinity. We used singly-primed M13 DNA as a template, with 9 – 576 nmol of POLG1 and 4-fold molar excess of POLG2. The density of the distinct bands was analysed with ImageQuant software (version 5.2, GE Healthcare, Piscataway, New Jersey) and K_d values calculated with Origin 7.5 (OriginLab) from the fraction of DNA bound. At least two independent experiments were done, with duplicate samples per enzyme.

6.6.3 ANALYSIS OF PRODUCTS OF PROCESSIVE DNA SYNTHESIS

We analysed the products of processive DNA synthesis by using singly-primed M13 DNA as a template, 20 ng of POLG1 and 4-fold excess of POLG2. The density of the bands was determined by computer integration analysis using ImageQuant software (version 5.2, GE Healthcare). We normalised the density of the bands to the nucleotide level to correct for the uniform labelling of the DNA products and the DNA strand lengths are presented as average processed units (APU), in nucleotides.

6.7 OVEREXPRESSION OF *POLG1* IN CELLS (III)

We produced four *POLG1* cDNAs with PCR site-directed mutagenesis: wild type *POLG1*, c.2243G→C *POLG1*, c.3428A→G *POLG1* and c.2243G→C together with c.3428A→G *POLG1*. Mutated PCR products were purified with DpnI restriction endonuclease (NEB), digested with EcoRI and BamRI restriction endonucleases and then gel purified. Blunt-ended *POLG1* inserts were ligated into pBABEpuro vectors and verified with sequencing. These vectors were used to transfect retrovirus packaging cells of GP+E86 cell line. We used puromycin as a selection agent, collected the medium and infected a PA317 cell line with the medium. Again, puromycin was used as a selection agent and virus-containing medium from PA317 cells was collected. Finally human primary fibroblasts were transduced with this medium. Infected, *POLG1* overexpressing cells were collected using standard trypsin-based method, and RNA was extracted from cells with Qiagen RNeasy Mini Kit (Qiagen). After reverse transcription of RNA, we did the real-time quantitative PCR analysis of *POLG1*, *C10ORF2* and, as an internal control, the beta actin gene *ACTB* using the TaqMan system (Invitrogen).

6.8 PULSE LABELLING OF MITOCHONDRIAL TRANSLATION PRODUCTS (III)

We studied the effect of overexpression of the above described mutant *POLG1*s in mitochondrial translation by first stopping the translation in cells, then labelling the cells with ^{35}S, stopping the labelling with centrifugation, isolating mitochondria and loading sonicated products to gradient gels. These gels were fixed, dried and exposed to Phosphor Screen, as described by (Boulet et al., 1992).

6.9 PROTEIN MODELLING OF POLG (III, VI)

We used VMD software to study the docking of DNA templates to POLG1 (PDB entry 3IKM, chain A) (Humphrey et al., 1996). We collected all known Alpers' disease mutations by using Human DNA Polymerase Gamma Mutation Database (http://tools.niehs.nih.gov/polg/). We collected a list of all *POLG1* mutations linked to Alpers' disease from the database and reviewed the references manually.

7 RESULTS AND DISCUSSION

7.1 CLINICAL MANIFESTATIONS OF MIRAS (I, II)

The clinical manifestation of MIRAS is not homogeneous: instead, there is great variability in almost all major features, such as age of onset, disease progression and epilepsy. However, no dominant inheritance of MIRAS has been described. Our MIRAS patient sample comprised a mother-son pair, both with a MIRAS phenotype, raising the question of a dominant SCA-disorder. The son also had an earlier age of onset for the symptoms, suggesting anticipation, but otherwise his disease progression resembled his mother's. In addition, we examined a sporadic MIRAS patient. Both patients, mother and son, were homozygous for MIRAS mutations (c.2243G→C and c. 3428A→G). This prompted us to examine *POLG1* of the father, who was asymptomatic. He was a carrier of the MIRAS disease allele. The carrier frequency of this allele is high in Finland, Norway and Belgium and introduction of three disease alleles, two from one parent and one from another, is a realistic possibility (Hakonen et al., 2007a; Hakonen et al., 2005; Van Goethem et al., 2003; Winterthun et al., 2005). Hereditary ataxias with dominant inheritance are usually thought to be spinocerebellar ataxias. However, atypical features such as epilepsy should guide the clinician to consider the possibility of MIRAS. Also, in countries where the carrier frequency of the MIRAS allele is high, *POLG1* should be considered even in the case of dominant inheritance.

The clinical features of MIRAS in the mother and son suggested anticipation and we wanted to study if it was caused by mtDNA alterations. mtDNA is maternally inherited and possible transmission of mtDNA deletions or point mutations could explain the earlier onset of MIRAS in the son. We examined the point mutation load and mtDNA copy number from these two MIRAS patients. Both patients had moderate mtDNA depletion, but no significant increase in point mutation load and no deletions in mtDNA. Although both patients shared some unique point mutations, we cannot conclude that mtDNA point mutation load would be a factor for the seemingly anticipatory clinical phenotype.

7.2 NEUROPATHOLOGY OF POLG1-PD AND MIRAS PATIENTS (I, II)

The clinical phenotypes of *POLG1* mutation patients vary from late-onset PEO phenotypes to early-onset MIRAS with catastrophic epileptic seizures,

among several others manifestations. We did a complete neuropathological examination of several *POLG1* patients, in order to understand the clinical manifestations and heterogeneity of the phenotypes. A specific area of interest was mesencephalon: POLG1-PD patients have PEO and parkinsonism and although sporadic reports of *Twinkle* mutation and MIRAS patients with parkinsonism exists, parkinsonism is not a common feature in patients with other alterations in mtDNA replication. Thus, we focused on the mesencephalic changes and asked if they could explain the parkinsonism.

The neuropathological features of the patients are only briefly summarized here. The full description of patients can be found in the original articles.

7.2.1 MIRAS

Neuropathological findings of our MIRAS patients were similar: highly specific sensory nervous system manifestation. Peripheral nerves showed subtotal loss of large myelinated fibers; spinal cord had severe neuron loss in posterior spinocerebellar tracts and posterior columns and in cerebellum loss of Purkinje cells and atrophy of dentate nucleus. Inferior olives had neuron loss with spongiotic vacuolization in mediodorsal thalamic nucleus and parieto-occipital region of subcortical white matter in one patient. One patient had several COX negative fibers, whereas one patient did not have any changes suggesting mitochondrial disease in her muscle biopsy sample. The neuropathological findings were confined to the sensory and proprioception system, which is in line with the previously published neuropathological examinations of MIRAS (Rantamäki et al., 2001; Van Goethem et al., 2004). Both patients also had moderate neuron loss in the pars compacta of SN, especially in the dorsal tier, but no clinical sings of PD. They did not either have Lewy bodies in the SN or mesencephalon. In PD at least 60% of SN neurons need to die, before the PD motor symptoms develop [Reviewed in (Braak et al., 2004)]. We know that POLG1 patients with a catalytic deficiency may develop parkinsonism and a sporadic MIRAS patient has been described with late onset parkinsonism (Luoma et al., 2004; Remes et al., 2008). Our results suggest that although MIRAS patients have neuron loss in the substantia nigra, parkinsonism is not a common feature. A question that cannot be answered in this thesis is whether MIRAS patients would eventually have developed parkinsonism, if they had lived longer.

7.2.2 POLG1-PD

We studied here the neuropathology of the mesencephalon of two of the originally described POLG1-PD patients and other mtDNA maintenance

disorders patients (MIRAS, IOSCA and Twinkle-PEO) without signs of PD, to ask whether correlation in genotype and phenotype could be seen at the neuropathological level. We wanted to understand if parkinsonism is a feature caused by mutations in the catalytic domain of *POLG1* or do other mutations, such as spacer mutations, also cause parkinsonism? Thus, we asked if neuropathological features could explain and verify the differences in the clinical phenotype of *POLG1* and also *Twinkle* patients.

The most drastic neuropathological finding in both patients was the almost complete destruction of the mesencephalon and especially SN and oculomotor nucleus. In both patients, there were just a few intact pigment neurons remaining in pars compacta of SN. In the oculomotor nucleus the destruction was not as complete, but still substantial. However, neither patient had Lewy-bodies in immunohistochemical stainings against p62, TAU or α-synuclein. Both patients had moderate neuron loss in posterior spinocerebellar tracts, cerebellar cortex, dentate nucleus and inferior olives. The thalamus was also mildly affected. In muscle biopsy samples both had typical findings for mitochondrial disease: COX negative fibers and mtDNA deletions. In comparison, we studied the mesencephalon in MIRAS, Twinkle PEO and IOSCA patients, who did not have parkinsonism. These patients had moderate (MIRAS, Twinkle PEO) and mild (IOSCA) loss of neurons in pars compacta of SN and oculomotor nucleus and no signs of Lewy-bodies.

Occasional dominant PEO-families have been reported with parkinsonism, but in 2004 these cases were shown to be caused by POLG1 mutations, indicating that POLG1 defects can cause parkinsonism (Chalmers et al., 1996; Checcarelli et al., 1994; Luoma et al., 2004; Moslemi et al., 1999; Siciliano et al., 2001). Not all POLG1 mutations show this phenotype, e.g. spacer mutations. Our POLG1-PD patients exhibit a similar phenotype as published cases of ARJPD: earlier onset of parkinsonian symptoms than in idiopathic PD, good response to levodopa treatment, no cognitive impairment and slow progression of the disease course (Albanese et al., 2005; Dekker et al., 2003; Ibáñez et al., 2006; Lücking et al., 2000; Mori et al., 1998; van de Warrenburg et al., 2001)..

Neuropathological findings of ARJPD also resemble the changes seen in our patients: no Lewy-bodies, no α-synuclein pathology and neuron loss limited to SN. In idiopathic PD, neuropathological changes also develop in other regions of the CNS than in mesencephalon [Reviewed in (Braak et al., 2004)]. In fact, the first areas affected are not in mesencephalon, but in the dorsal motor nucleus of the vagal nerve from which the Lewy neurites branch into the Locus Coeruleus, Raphe nucleus and finally to the SN. When the first changes in the SN can be seen, the motor symptoms also develop. In the later course of idiopathic PD, changes develop widely in the CNS, for example, into the primary sensory and motor fields. The neuropathological changes in

idiopathic PD begin in limited areas and finally affect the whole brain. In ARJPD, this feature has not been reported and instead the patients have very limited loss of neurons in the SN. This progressing destruction was not seen in our patients: although our patients also had neuron loss in other parts of the CNS, they did not exhibit the typical PD progression. The clinical and neuropathological findings in POLG1-PD resemble ARJPD more than idiopathic PD and we consider POLG1-PD and ARJPD to be part of the same group, namely mitochondrial PD.

Our initial hypothesis was that the mesencephalon and especially SN would be affected only in patients with parkinsonism. All mtDNA disorder patients examined had at least minor neuron loss in the SN. In oculomotor nucleus, the changes were not evident in every patient, as only Twinkle PEO and POLG1-PD patients had marked neuron loss in the oculomotor nucleus whereas IOSCA and MIRAS patients did not have any neuron loss in their oculomotor nucleus. In our patients the mitochondrial dysfunction is caused by a malfunction of mtDNA replication. In POLG1-PD, the defect is catalytic, whereas in MIRAS and IOSCA the catalytic activity of POLG or TWINKLE, respectively, is not affected (Hakonen et al., 2008a). In addition, MIRAS and IOSCA have tissue specific manifestations of mtDNA abnormalities, suggesting other regulating factors, which may affect the disease pathogenesis and ultimately the clinical phenotype. SN is a region of the brain, which is especially susceptible to even a small disruption in energy metabolism: SN neurons are distinct from other neurons in that they have enormous terminal projection fields, directly influencing ~75000 striatal neurons. Their axons are long, thin and have a poor myelin sheath and they have high energy expenditures [Reviewed in (Braak et al., 2004)]. Gene expression studies in rats suggest that energy metabolism transcripts are highly expressed in the SN, supporting the view that the SN neurons have high demands for energy metabolism and even a small disruption in this can lead to parkinsonism (Greene et al., 2005). However, the reason why only some patients with deficiency in mitochondrial DNA replication develop parkinsonism remains unknown.

7.3 ANALYSIS OF RESPIRATORY CHAIN IN MESENCEPHALON (II)

CI deficiency is a well-established feature in PD patients that has been described in multiple tissues and can be described as a widely accepted starting hypothesis in PD research (Bindoff et al., 1989; Haas et al., 1995; Krige et al., 1992; Parker et al., 1989; Schapira et al., 1989; Yoshino et al., 1992). Also, CI inhibitors, such as rotenone and MPTP cause parkinsonism and can be used to produce animal models for PD. In addition, MPP+, the neurotoxic ion of MPTP, affects the mtDNA structure by destabilizing the D-

loop structure, thus linking the neurotoxicity, mtDNA metabolism and parkinsonism (Miyako et al., 1999; Umeda et al., 2000). We wanted to study if the parkinsonism in POLG1-PD patients could be explained by a respiratory chain deficiency. We analysed the respiratory chain components by immunohistochemistry in the formalin fixed mesencephalon samples from patients: two POLG1-PD, three MIRAS, one IOSCA, one Twinkle-PEO patient and two healthy controls. All patients had a clear and dramatic decrease in CI immunoreactivity in the pigment neurons of SN (Figure 8). In the oculomotor nucleus, the decrease in CI immunoreactivity was evident in one POLG1-PD, MIRAS and Twinkle-PEO patient. The lack of CI immunoreactivity did not correlate with the age of onset, phenotype or age of the patients. We examined if the CI deficiency was associated with active proteolysis by analyzing the amount of a mitochondrial matrix-facing AAA-protease, AFG3L2, in all patients. AFG3L2 immunoreactivity was present in the neurons of SN and oculomotor nucleus of all patients, with slightly more intense staining in MIRAS and POLG1-PD patients. No findings of respiratory chain deficiency in the mesencephalon have been reported previously in POLG1 or Twinkle patients, but we know that in MIRAS and IOSCA CI deficiency is found in the neurons of the cerebellum and frontal cortex (Hakonen et al., 2008b). In a recent study of idiopathic PD patients and single SN neurons, CI deficiency was however limited to SN neurons that did not have Lewy pathology (Reeve et al., 2012). Our results suggest that CI deficiency is a feature of disease caused by malfunction of mtDNA replication proteins, POLG or Twinkle, not a feature of POLG1-PD as such. Our results challenge the long-standing assumption that CI deficiency is causative for PD.

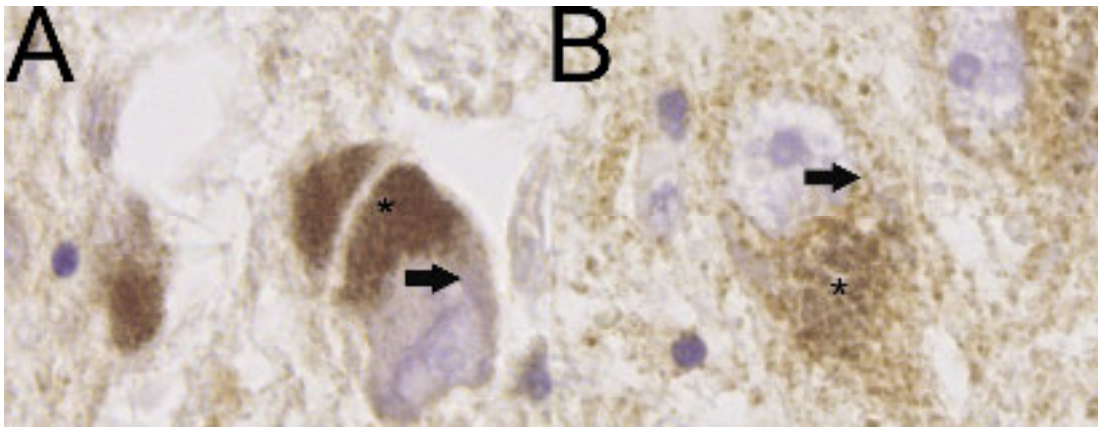

Figure 8 Complex I in substantia nigra pigment neurons. Asterisk marks the normal pigment. A: POLG1-PD patient, a clear lack of complex I immunoreactivity (arrow). B: Healthy control. Normal complex I immunoreactivity in the neuron (arrow). NDUFS3 antibody. Brown: CI immunoreactivity, blue counterstaining.

POLG-PD1 patients also had an increase in CII immunoreactivity in the SN pigment neurons, whereas MIRAS, IOSCA or Twinkle-PEO patients did

not have this change. The relevance of this increase remains unknown: CII might be upregulated as a protective mechanism in CI dysfunction. CII is the second entry point for electrons in the respiratory chain and increase in CII immunoreactivity raises the question whether substrates entering respiratory chain at CII are favored in these neurons. A reason for this might be that CI is a major ROS producer in mitochondria. If CI dysfunction leads to an increase in ROS production and thus mitochondrial stress, downregulation of CI and upregulation of CII might in fact be a protective factor against mitochondrial stressors. Another explanation is that CII is in this case a marker for mitochondrial quantity and increase in CII immunoreactivity tells us that the amount of mitochondria is increased as a compensatory mechanism.

We also examined the immunoreactivity against CIII and CIV. None of our patients had any changes in CIII or CIV immunoreactivity, in SN or oculomotor nucleus.

7.4 FUNCTIONAL AND STRUCTURAL ANALYSIS OF P.TRP748SER POLG1 (III)

MIRAS is caused by two nucleotide changes in *POLG1*: c.2243G→C in cis with c.3428A→G, resulting in p.Trp748Ser + p.Glu1143Gly amino acid changes. C.2243G→C is a disease causing mutation, whereas c.3428A→G is considered to be a polymorphism, with a frequency of ~3% in the general Finnish population. Previous work in our lab has showed that c.2243G→C in cis with c.3428A→G allele arose from a common European founder (Hakonen et al., 2007a; Hakonen et al., 2005). The clinical manifestations of MIRAS are severe, but the disease itself shows great heterogeneity in age of onset and other features. MIRAS mutations are also a known cause of Alpers' disease, widening the clinical spectrum even more. We wanted to study the biochemical phenotype of p.Trp748Ser in POLG1, to understand why this amino acid change is associated with so many phenotypes and produces variable clinical manifestations. We produced the recombinant mutant enzyme using a baculovirus expression system. We purified in total two exonuclease deficient proteins: wild-type and p.Trp748Ser POLG1. The exonuclease activity was eliminated by two amino acid substitutions in the exonuclease domain of POLG1, as described previously (Longley et al., 1998a). Exonuclease deficient POLG1 was used, as we knew from previous studies that the exonuclease activity of POLG1 might mask a deficiency in DNA polymerase activity (Graziewicz et al., 2004). Both enzymes were purified twice, in independent purifications, 180-fold with a final yield of 6%. All protein preparations were of similar purity and we did not find any signs of structural instability in any purified protein.

Table 4. *Catalytic properties of mutant POLG1 enzymes. p.Trp748Ser vs wild type POLG1, data in last column adapted from (Chan et al., 2006)*

	Wild type POLG1	**p.Trp748Ser POLG1**	**p.Ala467Thr POLG1**	**p.Trp748Ser vs wild type POLG1, (Chan et al.)**
Specific activity (units/ mg)				
Gapped double-stranded DNA	8100- 9300	7200- 26 000	3900	-
Poly (rA)- oligo (dT)	712 ± 121	852 ± 209	-	2.3%
Singly-primed M13 DNA	123 ± 14	143 ± 17	-	-
Km (µM dNTP)				
Gapped double-stranded DNA	66.1 ± 6.3	66.5 ± 4.8	-	-
Singly-primed M13 DNA	0.77 ± 0.07	0.75 ± 0.06	-	-
Poly (rA)- oligo (dT)	-	-	-	6 %
Average processive unit (nt)				
POLG1	52.8 ± 13.6	48.2 ± 12.7	55.8 ± 13.4	-
POLG holoenzyme	245 ± 34.0	257 ± 32.8	144 ±26.9	-
DNA binding affinity, Kd (nM)	6.8 ± 0.37	11.1 ± 0.52	-	-
DNA binding affinity, catalytic core	-	-	-	8-fold decrease

We did our initial analysis of DNA polymerase activity using gapped, natural sequence dsDNA as a template. We used a third, previously purified

and published p. Ala467Thr POLG1 mutant enzyme as a control for a catalytic defect (Luoma et al., 2005). We measured the specific activities to be 8100-9335 U/mg, 7200-26000 U/mg and 3900 U/mg, for wild-type, p.Trp748Ser and p. Ala467Thr respectively (Table 4). As this initial analysis revealed normal p.Trp748Ser DNA polymerase activity and we were able to replicate the previously published catalytic defect of p. Ala467Thr POLG1, we wanted to study the processive DNA polymerase activity next. We know from previous work, that POLG1 is able to use various ptDNAs and the activity of the enzyme is higher with templates with high primer density. To understand if p.Trp748Ser POLG1 DNA polymerase activity would be diminished with more challenging ptDNA, we used two different primer templates: singly-primed, natural sequence M13 DNA and multi-primed, artificial sequence poly (rA)·oligo(dT). For M13 DNA, the specific activities were 123 ± 14 and 143 ± 17 U/mg for wild-type and p.Trp748Ser POLG1. For poly (rA)·oligo(dT), the specific activities were 712 ± 121 and 852 ± 209 U/mg, respectively. The analysis of DNA product strand lengths with denaturing gel electrophoresis confirmed our results from different primer-templates: wild-type POLG1 had an average processed unit (APU) of 52.8 ± 13.6 nt, p.Trp748Ser POLG1 48.2 ± 12.7 nt and p. Ala467Thr POLG1 55.8 ± 13.4 nt. The steady state kinetics of the catalytic core, on gapped, natural sequence DNA did not reveal any significant differences: the K_m values were 66.1 ± 6.3 and 66.5 ± 4.8 µM dNTP for wild-type and p.Trp748Ser POLG1. In conclusion, we studied the DNA polymerase activity of p.Trp748Ser POLG1 using multiple templates and tests. None of them revealed any decrease in the DNA polymerase activity of the p.Trp748Ser POLG1. The lack of a DNA polymerase defect can question the possible pathogenicity of p.Trp748Ser POLG1, but we have previously published two other disease-related spacer mutants, p. Arg627Gln, p. Arg627Trp, which did not exhibit a biochemical phenotype. Also, the MIRAS disease allele is not found as a homozygote in healthy controls. The lack of catalytic deficiency is not a specific feature of MIRAS: in IOSCA, the disease causing mutation in Twinkle does not affect the catalytic activity of the TWINKLE helicase (Hakonen et al., 2008a).

In previous biochemical characterizations of the POLG1 spacer region mutant, p. Ala467Thr, both Luoma and co-workers and Chan and co-workers reported a remarkable decrease in polymerase activity and interaction with the accessory subunit, POLG2 (Chan et al., 2005; Luoma et al., 2005). This prompted us to study the function of the POLG holoenzyme. The holoenzyme was reconstituted with the same POLG1 enzymes as in other assays and four-fold molar excess of POLG2 accessory subunit, with optimal reaction conditions for holoenzyme. Again, we used M13 DNA for the initial analysis of steady state kinetics. Both wild-type and p.Trp748Ser POLG holoenzyme had similar K_m values: 0.77 ± 0.07 and 0.75 ± 0.06 µM dNTP. Next, we measured the DNA binding capability of the holoenzyme with quantitative electrophoretic mobility-shift with a 21/45-mer DNA as a primer-template.

The reconstituted wild-type POLG holoenzyme had a Kd of 6.8 ± 0.37 nM whereas p.Trp748Ser POLG holoenzyme had a slightly lower affinity, 11.1 ± 0.52 nM. The biochemical characterization of p.Trp748Ser POLG1 was completed with the measurement of the processivity of the holoenzyme. There was no significant change in the average processed units (APU): 245 ± 34.0, 257 ± 32.8 and 144 ±26.9 nt for wild type, p.Trp748Ser and p. Ala467Thr POLG holoenzyme, respectively. The holoenzyme processivity was ~5 fold higher than the catalytic core alone with wild-type and p.Trp748Ser POLG holoenzyme, whereas p. Ala467Thr POLG1, with a known defect in subunit interaction, was stimulated only ~2.6 fold when compared to the processivity of the catalytic core alone. We can conclude that the subunit interaction of p.Trp748Ser POLG1 was not affected and cannot explain the clinical phenotype.

The recently published crystal structure of human POLG1 enabled us to study the location of p.Trp748 and p.Glu1143 amino acids on the tertiary structure of POLG1 and explain the lack of biochemical phenotype in our assays (Lee et al., 2009). The p.Trp748 amino acid maps to a cluster on the tertiary structure of POLG1 that does not take part in DNA synthesis, but is predicted to affect the interaction with other mtDNA replication proteins, mtSSB (Figure 9). Two other Alpers' disease amino acid changes, which do not cause a biochemical phenotype, are also located in this cluster: p. Arg627Trp/Gln. To date, no biochemical defect has been reported in the interaction of human POLG and mtSSB, caused by POLG1 amino acid changes. However, human mtSSB amino acid changes lead to diminished POLG stimulation (Oliveira and Kaguni, 2010; Oliveira and Kaguni, 2011). Chan and co-workers have published a biochemical study of p.Trp748Ser, p.Glu1143Gly and p.Trp748Ser + p.Glu1143Gly POLG1 (Chan et al., 2006). They reported a severe DNA polymerase defect in p.Trp748Ser POLG1, which was partially rescued by the p.Glu1143Gly amino acid change. Their data suggested that the deficiency was caused by a weakened DNA binding capability of p.Trp748Ser POLG1. However, based on our careful biochemical analysis of p.Trp748Ser POLG1 and analysis of POLG1 crystal structure we conclude that p.Trp748Ser POLG1 does not affect the intrinsic DNA polymerase or DNA binding ability and the defect reported by Chan and co-workers is most likely artefactual.

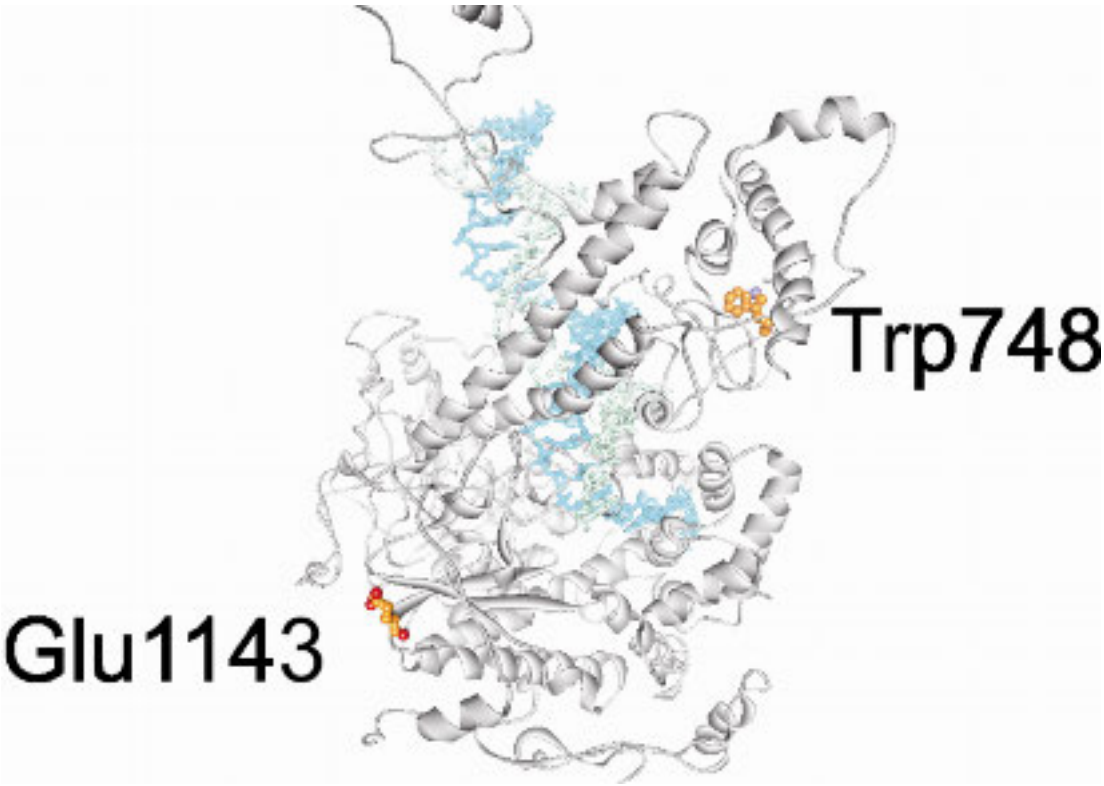

Figure 9 Location of p. Glu1143 and p. Trp748 amino acids in POLG1. Blue double strand: primer-template DNA. p. Trp748 and p. Glu1143 amino acids are located in different parts of the POLG1 enzyme and neither of them is involved directly in DNA binding.

The role of the putative polymorphism c. 3428A→G, leading to amino acid change p.Glu1143Gly in POLG1 has been unclear. The structural data shows that p.Glu1143 amino acid maps near amino acids that are located in the pol domain of POLG1 (Figure 10). However, this amino acid is located at the outer edge of the enzyme, not in the vicinity of the pol site. Defects in this structure are predicted to reduce the polymerase rate of POLG1, based on their distances from the pol active site (Kasiviswanathan et al., 2009). Secondly, p.Glu1143 is in a different part of the POLG1 enzyme than p.Trp748Ser. The distance between these amino acids is ~61.5 Å. In yeast, the equivalent amino acid change leads to a decrease in polymerase activity, due to structural instability of the POLG1 enzyme (Baruffini et al., 2007). We find it improbable that it would affect the DNA polymerase activity, let alone rescue a possible defect caused by an amino acid change in a different part of the enzyme. The p. Gln1236His amino acid change, caused by c. 3708 G→T mutation in *POLG1*, also leads to an increase in POLG1 function. p. Gln1236His is thought to be a polymorphism and is found *in cis* with the p. Arg627Gln amino acid change (Luoma et al., 2005). Although it is possible that these polymorphisms give the mutant enzyme an evolutionary advance over single amino acid changes, we find it highly unlikely that they would rescue an enzyme lacking nearly all activity.

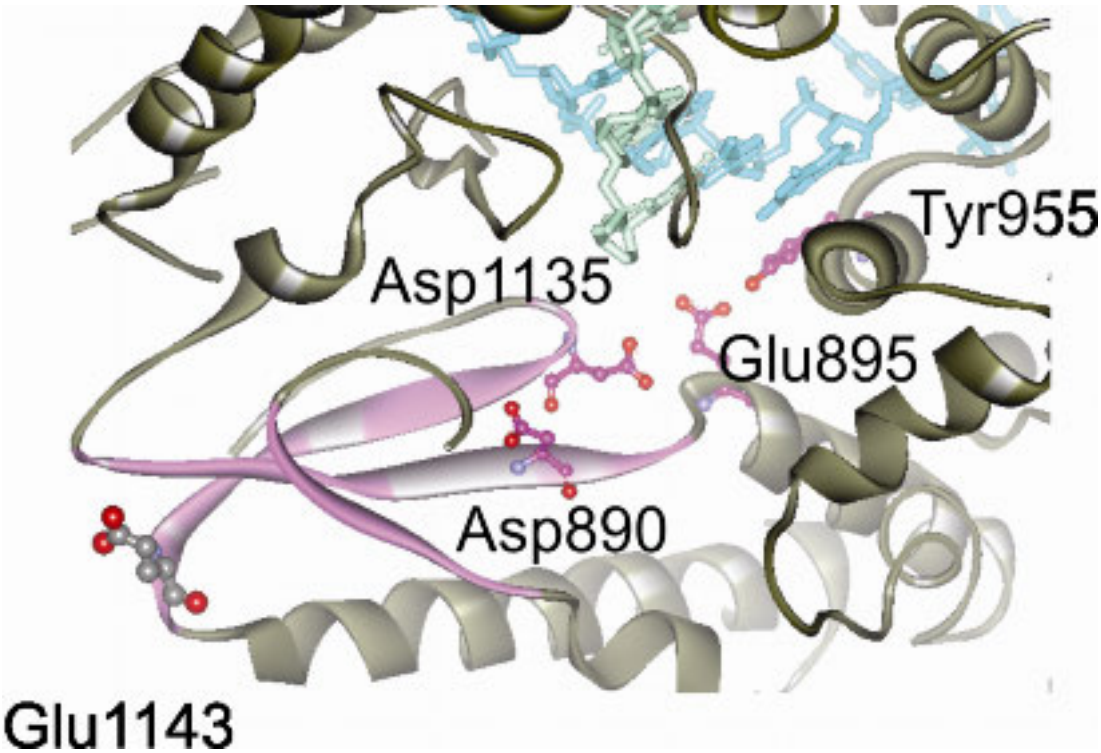

Figure 10 Relationship of p. Glu1143 and the pol active site. Blue double strand: DNA. Pink sheets: Beta-sheets. Tyr955 is located in the pol active site. Glu1143 is the most lateral amino acid in the structure formed by the three beta sheets. Mutations in these beta-sheets result in the reduction of DNA polymerase activity, but the long distance between Glu1143 and pol active site make it unlikely that Glu1143 would affect the polymerase activity of the enzyme in increasing manner.

7.5 OVEREXPRESSION OF C.2243G→C MUTANT *POLG1* IN CELLS (III)

We created a cell-based, retroviral overexpression system to be used as a modelling tool to study the effect of the *POLG1* c.2243G→C, c.3428A→G and c.2243G→C in *cis* with c.3428A→G mutants. We achieved a high level of *POLG1* overexpression in primary cells with all retroviral vectors: with quantitative RT-PCR, the overexpression was 7-19 fold higher, when compared to endogenous expression levels. We also measured the expression level of *Twinkle* and found no change. Neither mtDNA levels nor mitochondrial translation were affected by the overexpression of wild type or mutant *POLG1*. Although this system worked properly, we were unable to use it in the analysis of the mutations, due to the lack of downstream changes. This was probably due to the remaining endogenous wildtype POLG, which was likely able to compensate the functional defect caused by recessive p.Trp748Ser POLG. The same phenomenon has been described also for another recessive POLG1 amino acid change, p. Gly848Ser (Atanassova et al., 2011).

7.6 CLUSTERING OF ALPERS' DISEASE MUTATIONS IN POLG1 (III, IV)

Alpers' disease is a recessive disease, caused by multiple different compound heterozygous or homozygous mutations in *POLG1* or *Twinkle* (Naviaux and Nguyen, 2004; Sarzi et al., 2007). The large number of known causative mutations for Alpers' disease and other diseases caused by *POLG1* mutations makes it difficult to analyse the biochemical deficiency manually, as the characterization of each different mutant enzyme requires construction and purification of altered protein, which is both a time consuming and error prone operation. In addition, the standard measurement of DNA polymerase activity, processivity, DNA binding affinity and interaction with POLG2 does not necessarily exhibit a clear biochemical phenotype, as is the case with p. Trp748Ser amino acid change. Also, cell culture based systems cannot be used reliably in the analysis of POLG activity because of our inability to block the activity of other DNA polymerases reliably. Thus, we wanted to produce a robust tool to examine the possible pathogenicity of the current and new mutations in the POLG1. Utilizing the three dimensional model of POLG, based on the crystallization of the protein, we examined the crystal structure of human POLG in its apoenzyme form (Lee et al., 2009). We also utilized and combined the biochemical data accumulated during the last decades from *D. melanogaster*, *S. Cerevisiae* and human mutations with the published crystal structure of POLG. This extensive combination of data indicated that the mutations in linear DNA and protein structures of POLG1 cluster within the tertiary structure to five distinct clusters, despite no apparent clustering to previously known protein clusters having been seen.

Cluster 1 residues affect the polymerase activity either via pol active site, DNA binding channel or the surrounding architecture. POLG1 p. Tyr955Cys amino acid change causes adPEO with parkinsonism, with multiple mtDNA deletions (Figure 5) (Van Goethem et al., 2001). This change locates to the pol active site (Figure 10). The biochemical phenotype caused by this amino acid change is dramatic: almost complete lack of the polymerase activity, error-prone DNA synthesis and sensitivity to dNTP concentration (Graziewicz et al., 2004; Ponamarev et al., 2002). The multiple mtDNA deletions are explained by sensitivity to dATPs: in low dATP concentration p. Tyr955Cys POLG1 exhibits stalling phenotype at dATP insertion sites (Atanassova et al., 2011). Other amino acid changes in this cluster do not necessarily exhibit as clear biochemical phenotype. For example, p. Thr885Ser amino acid change, which causes Alpers' disease, is located near the pol active site, in the adjacent beta-sheets. This change reduces the DNA polymerase activity two-fold. Other changes in the same beta-sheet structure reduce also DNA polymerase activity, with the reduction being more severe the closer the change is to pol active site (Kasiviswanathan et al., 2009).

These two changes underline the different biochemical and clinical phenotypes caused by changes in the same functional domain of POLG.

Cluster 2 is composed of residues that interact with the upstream DNA duplex. These residues are part of the spacer domain. The p. Ala467Thr is probably the most common disease-causing amino acid change in POLG1 and is predicted to locate within cluster 2 (Figure 5). In *D. melanogaster*, a triple change of amino acids p. Pro556Ala, p. Lys557Ala and p. Ile558Ala disrupts a hydrophobic structure, which shapes the DNA binding channel wall and leads to a complete lack of DNA binding and DNA polymerase activity (Luo and Kaguni, 2005). The amino acid change p. Ala467Thr in human POLG1 disrupts the same structure and also leads to a decrease in DNA binding and DNA polymerase activity, which is partially rescued by POLG2 (Chan et al., 2005; Luoma et al., 2005). These results are in line with the structural data, implying that the residues in this cluster affect the DNA polymerase activity due to changes in the DNA binding channel, and decreased processivity due to inability of accessory subunit to stabilize the DNA binding channel.

Cluster 3 residues form a structure for which we proposed the name "partitioning loop". Mutations in cluster 3 residues, for example the p. Leu304Arg, cause PEO, SANDO and Alpers' disease (Figure 5) (Tang et al., 2011). In yeast, the equivalent amino acid changes cause lack of processive DNA polymerase activity, low DNA binding affinity and increased 3'-5' exonuclease activity (Szczepanowska and Foury, 2010). These biochemical properties are explained by the structural data: the partitioning loop is proposed to modulate the partitioning of the primer strand between the pol and exo active sites. ptDNA that contains mispairs or lesions is destabilized and then led to a exo active site, whereas correctly paired ptDNA is stabilized. Malfunction of partitioning affects the exonuclease activity, thus explaining the biochemical phenotype of p. Leu304Arg POLG1.

Cluster 4 can be defined as residues around the residue p. Arg232, which affects the interaction with the distal POLG2. *POLG1* mutations leading to amino acid changes p. Arg232Gly and p. Arg232His cause Alpers' disease, which is explained by the biochemical phenotype of mutant enzymes: decreased DNA polymerase activity, DNA binding and disruption of exonuclease activity (Figure 5) (Ashley et al., 2008; Ferrari et al., 2005; Lee et al., 2010a). Structural data shows that the interaction between POLG1 and the distal accessory subunit is through one ion bond: this bond is between POLG1 p. Arg232 and POLG2 p. Glu394. Overall the distal accessory subunit increases the polymerization rate of the holoenzyme (Lee et al., 2010b). Thus, cluster 4 mutants affect the interaction between POLG1 and the distal POLG2.

Cluster 5 is located in the distal surface of the IP subdomain, distant from the DNA binding channel. Several important amino acid changes, leading to clinical phenotypes, map to this cluster: p.Trp748Ser, leading to MIRAS, p. Arg627Trp/Gln leading to Alpers' disease and others (Figure 5). The biochemical data from *D. melanogaster* support a disruption in the interaction between the POLG holoenzyme and mtSSB (Luo and Kaguni, 2005). The data also support the finding that residues in cluster 5 do not lead to a catalytic defect (Luoma et al., 2005).

Clustering of the Alpers' syndrome-causing mutations enabled us to ask what kinds of mutation combinations are allowed in compound heterozygous situations in Alpers' disease patients. One important finding in our study was that the compound heterozygous mutations map to different clusters. The conclusion from this is that although a single, recessive mutation as a homozygous mutation may cause a mild or non-existent biochemical phenotype, a combination of two alterations in different clusters of POLG1 leads to stronger clinical phenotype (Naviaux and Nguyen, 2004). Also, two different mutations in a single cluster would predictably lead to a drastic biochemical phenotype. However, no such combinations are reported in the literature, which suggest that such combinations are not tolerated. POLG holoenzyme is a heterotrimer, but we do not know if two holoenzymes work together, both replicating different strands of mtDNA. This would explain the more severe clinical picture of compound heterozygous mutations. A recent characterization of p. Tyr955Cys POLG1 examined the dominant effect of the mutations and found that in the presence of wild type POLG1 only a minor amount of full-length replication products are produced, due to the stalling of replication with p. Tyr955Cys POLG1, whereas with recessive p. Gly848Ser the addition of wild-type POLG1 rescues the phenotype (Atanassova et al., 2011). This result can be extrapolated to explain the more drastic phenotype of compound heterozygous mutations: no wild-type POLG1 exists and both mutant POLG1 enzymes accelerate the deficiency of POLG1 function via different mechanisms.

Currently, analysis of *POLG1* mutations is troublesome: the mutations cause different phenotypes and no reliable way to predict the possible pathogenicity or biochemical phenotype exists. At present, the pathogenicity of a new mutation is predicted by studying the possible absence in the normal population, conservation of the affected amino acid among species and segregation in the patient's family. The prediction tool that we have created, utilizing the 3D POLG1 structure and functional domain data, may aid clinicians in predicting the possible effect and pathogenicity of a new disease variant.

8 CONCLUSIONS AND FUTURE PROSPECTS

The work presented in this thesis provides new information about the pathogenesis and clinical manifestations of POLG disorders. We have found that all examined patients with an mtDNA replication disorder have neuronal loss in the SN and most importantly, that CI deficiency does not explain the parkinsonism in the POLG1-PD patients. Our results give insight into the clinical variety of POLG1 manifestations, based on the biochemical characterization of MIRAS POLG1 and structural analysis of POLG1 holoenzyme. Structural analysis of POLG holoenzyme and Alpers' disease mutations reveal new functional domains in the POLG structure, which may aid the clinician in the evaluation of new *POLG1* mutations.

In countries with a high carrier frequency of the MIRAS disease allele, a family history suggesting dominant inheritance should not be an exclusion criterion for a MIRAS diagnosis. We described a family in which both the mother and son had MIRAS, mimicking dominant spinocerebellar ataxia. The high carrier frequency of the disease allele had caused two separate introductions of the disease allele in the family and hence masked the recessive inheritance pattern.

CI deficiency of SN neurons was not the primary cause of parkinsonism in POLG1-PD patients, as we saw the same level of CI deficiency in MIRAS, IOSCA and Twinkle PEO patients, without correlation to age, age of onset or clinical phenotype of the patients. The neuron loss in SN correlated well with parkinsonism: POLG1-PD patients had an almost complete lack of neurons in the SN whereas MIRAS, IOSCA and Twinkle PEO patients, who did not have parkinsonism, had mild to moderate neuron loss in the SN. In our opinion neuronal CI deficiency is a general feature of the diseases caused by malfunction of the mtDNA replication machinery and whether it contributes to pathogenesis or is a physiological response remains to be studied. We do not, however, understand what causes the tissue specific changes, or why Twinkle and POLG1 disease have such heterogeneity in their symptoms and disease progression. Patients with PEO and parkinsonism, caused by *Twinkle* mutations have been described in the literature, but no neuropathological examinations exist. This would help to understand the variability in the manifestation of parkinsonism in PEO patients. Also, no animal models of POLG1-PD, MIRAS or IOSCA exist currently and to understand the tissue specific changes and disease progression, an animal model would be an ideal tool.

Our structural analysis of POLG1 suggests that p.Trp748Ser, a common cause for MIRAS, participates in the interaction with mtSSB or Twinkle, and may thus affect the function of mtDNA replisome. This is supported by our biochemical characterization of p.Trp748Ser in POLG1, in which we did not see a direct effect on the catalytic activity or subunit interaction of POLG. Alteration in this interaction could lead to malfunction of mtDNA replication machinery. This hypothesis needs further investigation.

Recessive mutations in *POLG1* map into five clusters on the tertiary structure of POLG1. The functional profile of each cluster is predicted to be distinct: this is supported by the structural analysis and biochemical profiles of examined mutant proteins. We learned that in patients with compound heterozygous changes in *POLG1* the changes seem to map to different clusters and the lack of two different changes in the same cluster would suggest that such a combination would not be tolerated. Homozygous changes, such as p. Ala467Thr are an exception to this. However, patients with homozygous changes in POLG1 seem to have less severe clinical manifestation than patients with compound heterozygous changes, as is the case in MIRAS and Alpers' disease. This suggests that for an unknown reason the homozygous changes are tolerated more. Using the structural data and information about the clusters, clinicians might be able to predict the possible consequences of a new mutation. This is valuable, as the number of known *POLG1* mutations is increasing constantly and doing an individual functional assessment of each *POLG1* mutation is time consuming and error prone.

The current advances in sequencing techniques, such as whole-exome and whole genome sequencing have already provided us with multiple new disease loci for mtDNA replication diseases. This development will continue and most likely will be the basis for mitochondrial research for the next few years. It will not, however, provide us with the understanding of what causes the clinical heterogeneity of POLG1 or mtDNA replication disease in general. We know the basic mtDNA replication machine well and we understand how the individual components work. Despite this, we do not know how all the components function together. One explanation for the clinical heterogeneity is disruption in the function of the whole mtDNA replisome: the structural data suggests an interaction with POLG and mtSSB, which might be the disease causing disruption in MIRAS. The challenge is to understand the function of the whole mtDNA replication system and the factors affecting it, not just individual components. Maybe then we will have the ability to understand the varying clinical phenotypes.

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Helsinki, December 2012
Eino Palin

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